Development of endometrial fibrosis in the mare: Factors involved in tissue remodelling and collagen deposition

Charlotta Oddsdóttir

Submitted in fulfilment of the requirements of the degree of
Doctor of Philosophy

The University of Edinburgh
2007

Declaration

I declare that the research described within this thesis is my own work and that this thesis is my own composition

Charlotta Oddsdóttir
Edinburgh, 2007

Contents

Abstract

Age-related degeneration of the equine endometrium is an established and important cause of fertility problems in thoroughbred mares, causing great loss to the industry. As a part of the age-related endometrial degeneration complex, an excessive deposition of collagen leading to endometrial fibrosis is particularly important due to the limitations it causes to uterine function. The consequences include reduced efficacy of uterine defence mechanisms and a decrease in the uterine capacity for foetal nutrition.

Extensive research into the process of fibrosis in other organs has shown that this condition results from the malfunction of physiological tissue repair mechanisms. These mechanisms revolve around tissue fibroblasts that due to continuous stimulation secrete excessive amounts of collagen and inhibit the activation of factors essential to the normal collagen degradation occurring in scar resolution. Among these factors are the MMPs, an enzyme family with the ability to degrade extracellular matrix components such as collagen during the normal repair mechanisms following tissue injury. The malfunction in the regulation of these enzymes is important in the development of fibrosis in the liver and other organs.

In this study it was demonstrated that MMPs are involved in the acute uterine inflammatory response and that they were secreted by infiltrating inflammatory cells. The cellular mechanisms observed during endometritis in normal mares were comparable to the normal repair mechanisms known to be altered in the fibrosis of other organs. These enzymes were present in equine foetal fluids, and their regulation may be important in the process of abortion and stillbirth. It was demonstrated that inbreeding may be correlated with increased deposition of endometrial collagen in a study population of the Icelandic horse breed even though this breed appears to exhibit less severe endometrial degeneration than what is known in lighter breeds. It is likely that genetic predisposition leads to the disruption of normally self-limiting inflammatory and repair mechanisms in the endometrium, resulting in constant activation of collagen synthesis by local and infiltrating cells.

This thesis has shown that tissue repair mechanisms involving MMPs are likely to be involved in endometrial fibrosis in the mare. An inherent alteration in these mechanisms may play a role in the pathogenesis of this condition, and might arise due to genetic predisposition. Further understanding of the pathways leading to excess collagen amounts in the endometrium may produce preventative measures, and even therapeutic targets.

Acknowledgements

I would like to thank Elaine Watson and Simon Riley for their guidance and valued input during my research. I also want to thank Darren Shaw for his invaluable support, for giving his time on short notice, endless cups of coffee and good humour.

Rose Leask gets my sincere thanks for her unfailing support and friendship in the lab, and so do Paul Wright, Davey Pierce and all the people in the yard for helping me manage the mares and stallion. I am grateful to my friend and colleague Javier Aguilar who was extremely helpful with the clinical experiments as well as great company within and out of the country. I want to thank Lorna Hume for her assistance with microbiological cultures and Sionagh Smith for hours spent counting cells, and for being positive and supportive. Abigail Fowden and Sidney Ricketts receive my sincere thanks for providing me with samples for this study, as well as their learned advice.

I would like to thank my good friend and colleague Sigríður Björnsdóttir (Systa) who with her optimism and go-ahead attitude recruited the Icelandic mares and collected the samples and for her good advice and motivating influence. My friend Bea Wissmann gets my sincere thanks for her company throughout my time in Edinburgh, as well as verging on an all-nighter in supporting me in my final hours of writing this thesis. Limma and Nick and their family have been my family away from home for the past four years and it has been priceless to have their home as my home. My family in Iceland has been supportive and motivating throughout my studies and my mother gets my deepest thanks for always being there through the ups and downs. Takk, elsku mamma mín.

Finally, I am grateful for the Division of Veterinary Clinical Science scholarship and to RANNÍS (The Icelandic Research Fund for Graduate Students) for their financial support.

Abbreviations

AI	artificial insemination
BM	basement membrane
BSA	bovine serum albumin
CDE	chronic degenerative endometrial disease
CEM	contagious equine metritis
cfu	colony forming units
CIE	chronic infiltrative endometritis
CL	corpus luteum
DAB	3,3'-diaminobenzidine
eCG	equine chorionic gonadotrophin
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FSH	follicle stimulating hormone
GnRH	gonadotrophin releasing hormone
H&E	haematoxylin and eosin
HRP	horseradish peroxidase
HSC	hepatic stellate cell
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton
LH	luteinizing hormone

LT	leukotriene
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MT-MMP	membrane-type metalloproteinase
OCT	optimal cutting temperature
PBS	phosphate-buffered saline
PG	prostaglandin
$PGF_2\alpha$	prostaglandin $F_2\alpha$
pvc	polyvinyl chloride
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinases
TTBS	tween tris-buffered saline
uOD	units of optical density
TNF	tumour necrosis factor

List of figures

List of Tables

1 Chapter 1:

General introduction

1 Chapter 1: Introduction

Fertility problems cause major loss to the thoroughbred industry (Jeffcott *et al.* 1982; Morris & Allen 2002). The uterus of thoroughbred mares has functional limitations on foetal growth due to spatial constraints (Vandeplassche *et al.* 1970; Jeffcott & Whitwell 1973), that may result in pregnancy loss, placental insufficiency or the birth of small, weak foals (Bracher *et al.* 1996). These functional limitations may in older mares become exaggerated by a complex of age-related degenerative endometrial changes, known as chronic degenerative endometrial disease (**CDE**) or endometrosis, that is well documented in mares of various breeds (Ricketts 1975a; Doig *et al.* 1981; Ricketts & Alonso 1991; Troedsson *et al.* 1993a; Walter *et al.* 2001), but is particularly devastating to the thoroughbred industry. The degenerative changes include endometrial fibrosis that decreases the uterine capacity for foetal nutrition. In the already restricted thoroughbred uterus this may lead to delayed placentation (Bracher *et al.* 1996), and smaller foals with poor racing performance (Barron 1995).

Severe endometrial fibrosis has also been associated with accumulations of intrauterine fluid after breeding (Kotilainen *et al.* 1994), leading to increased embryonic death due to an embryo-hostile uterine environment (Carnevale & Ginther 1992). Intrauterine fluid accumulations have been observed on the day after breeding in 16% thoroughbred mares (Zent & Troedsson 1998) and pregnancy loss between Days 15 and 35 of gestation has been reported as the major loss to the thoroughbred industry (Morris & Allen 2002).

Various studies suggest that CDE is positively correlated with increased mare age (Chevalier-Clément 1989; Ricketts & Alonso 1991; Bracher 1997). However, this does not explain why some mares are much more severely affected than others at a comparable age (Ricketts & Alonso 1991). In a mixed-breed population of mares, it was concluded that the average age for beginning signs of CDE was ten years, and severe signs should not be seen in mares under 17 years of age (Ricketts & Alonso 1991). There is great commercial pressure to breed from older thoroughbred mares of proven genetic merit, but these mares may have already developed degenerative endometrial changes with resulting fertility problems.

Thoroughbred breeders are required to adhere to the regulations of the General Stud Book where it is not accepted to use artificial insemination and embryo transfer methods. In order to be registered as thoroughbreds for racing, foals born to thoroughbred mares are required to have been produced by natural coverage of a mare by a stallion (Weatherbys 2006). This restricts the choice of breeding methods that otherwise are available for the treatment of mares with fertility problems.

There are no known therapies for endometrial fibrosis (Van Camp 1993) and mares with severe irreversible endometrial fibrosis may be retired from breeding. Although the important consequences of endometrial fibrosis are well documented, a limited number of studies have been performed to determine the pathogenesis of the condition.

In other organ systems, such as the liver, the pathogenesis of fibrosis is considered to be an interruption in the balance between collagen deposition and breakdown (reviewed by Bataller & Brenner 2005; reviewed by Iredale 1997). The matrix metalloproteinases (**MMP**s) play an important role in collagen metabolism, and a disruption in their activity has been associated with fibrosis of the liver (reviewed by Iredale 1997), kidney (Jones *et al.* 1995) and other organs. Furthermore, a study using immunohistochemical methods on equine endometrium associated MMPs with areas of endometrial fibrosis (Walter *et al.* 2005). Understanding the pathogenesis of endometrial fibrosis, including the possible role played by matrix metalloproteinases could provide new clinical, diagnostic, and pharmacological procedures and interventions for this condition.

This thesis will attempt to explore some of the underlying mechanisms of endometrial fibrosis and aspects of tissue remodelling by MMPs that occurs during the normal oestrous cycle, as a consequence of uterine inflammation and during pregnancy.

1.1 Equine reproductive anatomy and physiology

1.1.1 Reproductive anatomy of the mare

The reproductive tract of the mare comprises the ovaries and the tubular reproductive organs; uterus, cervix, vagina and vulva. The uterus is a tubular organ made up of the body (corpus) and two short horns (cornua). The uterine wall is composed of three layers (**Figure 1.1**): the outermost serosal layer (perimetrium), the middle smooth muscle layer (myometrium) and toward the lumen is the mucosa (endometrium).

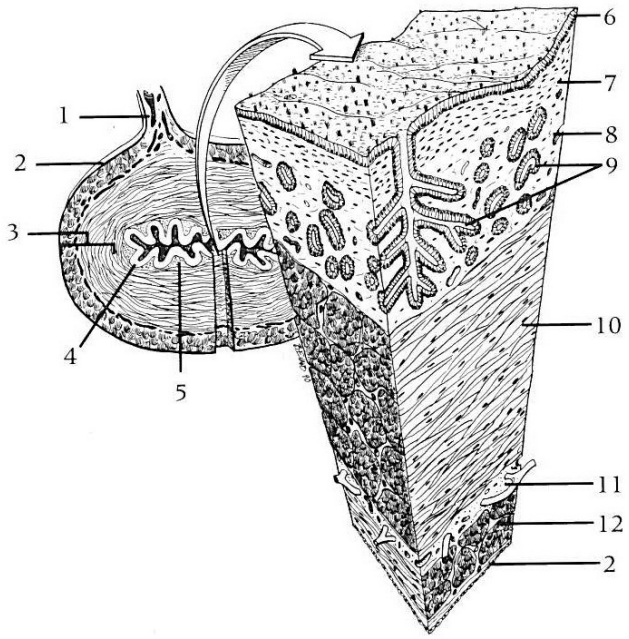


Figure 1.1 Cross section of equine uterine horn. 1, mesometrium; 2, perimetrium; 3, myometrium; 4, endometrium; 5, endometrial stroma; 6, luminal epithelium; 7, stratum compactum of lamina propria; 8, stratum spongiosum of lamina propria; 9, endometrial glands; 10, inner circular layer of myometrium; 11, middle (vascular) layer of myometrium; 12, outer longitudinal layer of myometrium (reproduced from Doig & Waelchli 1993).

The lumen of the uterus is virtually obliterated due to its flaccid walls and 12-15 large longitudinal endometrial folds, so only capillary spaces occur between the endometrial folds (Kenney 1978).

1.1.1.1 Histological structure of the equine endometrium

The equine endometrium is nondeciduate and is made up of two layers, luminal epithelium and lamina propria (**Figure 1.1**), that extends from the basement membrane (**BM**) of the epithelium to the myometrium. The lamina propria is further divided into two layers based on the density of stromal cells. The stratum compactum is immediately below the luminal epithelium and has a high density of stromal cells as well as capillaries. The stratum spongiosum has a low cellular density and a spongy appearance, due to numerous fibres connecting cells and many lymphatic vessels. Throughout the lamina propria are endometrial glands, derived from the luminal epithelium (Gray *et al.* 2001), and communicating with the uterine lumen via glandular ducts.

1.1.1.2 The role of endometrial glands

The glandular ducts play a vital role in the support of the equine conceptus, before and after placentation. The equine conceptus is relatively unattached for the first 35 days of gestation (see chapter **1.1.3**), and therefore relies on the endometrial glands for nutritional support (Samuel *et al.* 1977). During placentation, endometrial crypts intertwine with microvilli of the placenta, forming the microplacentomes (see **1.1.3.3**). The openings of the uterine glands are found in smooth areas of endometrium that lie between microplacentomes, where the chorioallantois is

separated from the underlying endometrium by the products of glandular secretions. The glands maintain a secretory function throughout the course of pregnancy, and are probably of considerable importance in the transmission of large water-soluble molecules from mother to foetus (Steven 1982).

1.1.2 Reproductive activity in the mare

Mares are seasonally polyoestrous and are reproductively active from spring to autumn, going into a phase of quiescence (anoestrus) during the winter months. Cycling activity depends on day length, nutrition and weather conditions. During the spring transitional phase when day length gradually increases, increased secretion of gonadotrophin releasing hormone (**GnRH**) induces the secretion of follicle stimulating hormone (**FSH**) to stimulate the growth of ovarian follicles (Sharp & Grubaugh 1987). Although the follicles reach a considerable size of around 30 mm in diameter, they may not ovulate but will regress due to the lack of luteinising hormone (**LH**), the initiator of ovulation (Davis, Grubaugh & Weithenauer 1987). For the secretion of LH to occur, the growing follicles have to be able to synthesise oestrogen. Once a follicle with the ability to secrete oestrogen reaches an ovulatory size of over 35 mm in diameter, the oestrogen will induce an LH surge to produce an ovulation (Mercer *et al.* 1988). Due to this ongoing development of ovarian follicles, the oestrous cycle may be irregular, but will usually normalise as spring advances and after ovulation has occurred. Similarly, the cyclical activity diminishes with decreasing day length in the autumn.

1.1.2.1 The oestrous cycle of the mare

The equine oestrous cycle is defined as the repetitive sequence of events that prepares the mare for conception, lasting on average 21 days. Oestrus is the period during which the mare is sexually receptive to the stallion, the ovulatory (dominant) follicle(s) develop and ovulation occurs. Oestrus lasts on average 6-7 days, with ovulation occurring 24 to 48 hours before the end of sexual receptivity, marking the start of dioestrus. Dioestrus is the period during which the mare is not receptive to the stallion and the genital tracts prepares for the reception and nurturing of the conceptus. During dioestrus the ruptured follicle develops into a corpus luteum (**CL**) that secretes progesterone and, in the non-pregnant mare, regresses 14-15 days after ovulation. Considerable follicular growth takes place in equine ovaries during the luteal phase that can result in normal fertile ovulations. The largest follicle at the time of luteolysis usually continues to enlarge and becomes the primary ovulatory follicle, reaching an average size of $\geq$ 45 mm in diameter during the last 24 hours prior to ovulation (Townson & Ginther 1989). The cyclical changes in the ovaries are controlled by gonadotrophins whereas the changes occurring in the tubular reproductive organs happen in response to progesterone and oestrogen secreted by the ovaries.

1.1.2.2 Oestrous cycle-dependent changes in the uterus

Under the influence of oestrogen during oestrus, the uterine wall thickens, muscular tone increases and vascularity becomes greater. Endometrial glands proliferate and become active and the lamina propria is highly oedematous, as is clearly seen on transrectal ultrasonography. The external os of the cervix is relaxed

and will allow the introduction of three or four fingers. During dioestrus, the influence of progesterone leads to a decrease in uterine wall thickness, myometrial tone and endometrial gland activity. The cervix becomes firmer and is tightly closed.

The stage of the oestrous cycle can be determined by the histological appearance of the luminal epithelium and stroma of the endometrium. During oestrus, luminal epithelial cells are tall columnar (20-30 µm) with cytoplasmic vacuoles in their basal third (Kenney 1978). Neutrophils can be seen to marginate in venules and capillaries, but they do not migrate through to the stroma. The presence of neutrophils in the stroma is therefore indicative of inflammation (Kenney 1978). Furthermore, oestrogen-induced oedema leads to reduced gland density and a loosely woven appearance of the stroma.

During dioestrus, luminal epithelium is low to tall columnar (10-15 µm), increasing in height (20 µm) approaching the next oestrus. Organelles increase in number and size in stromal cells and glandular epithelial cell, indicating active protein synthesis during this stage of the cycle (Ferreira-Dias *et al.* 1999). Gland density increases due to decreased stromal oedema and the increased tortuosity of glands. During winter anoestrus the endometrium undergoes some degree of atrophy, characterised by cuboidal epithelium and straight endometrial glands.

1.1.3 Events during equine pregnancy and parturition

Pregnancy in the mare lasts on average 335 to 342 days (Morris & Allen 2002) and involves communication events between the endometrium and conceptus

that are unique to the equine species, such as the prolonged mobility of the conceptus and formation of endometrial cups.

1.1.3.1 Fertilization, conceptus mobility and fixation

Fertilization occurs in the uterine tube, and the tubal phase of the embryo lasts 5-6 days, when it arrives in the uterus at the early blastocyst stage (Betteridge *et al.* 1982). At this stage, the embryo is surrounded by the zona pellucida, and during the next 48 hours the deposition of an electron-dense layer on the inside of the zona pellucida occurs (Strzemienski *et al.* 1984; Flood *et al.* 1982). The zona is eventually shed, leaving the embryo encased in this new covering, the capsule. The capsule is about 4 µm thick and forms a spherical, resilient and acellular envelope around the cellular components of the embryo, and is thought to have a role in the maternal recognition of pregnancy and uptake and transport of maternal proteins (Crossett *et al.* 1996). Soon after its arrival in the uterus, the conceptus embarks on a highly mobile phase, constantly moving between the uterine horns (Ginther 1983a; Allen & Bracher 1992). This ensures an extensive contact between the embryonic vesicle and the endometrium, thus preventing prostaglandin secretion by the endometrium, that otherwise would cause luteolysis (Ginther 1983a). The embryonic vesicle also induces an increased vascular perfusion of the endometrium in the pregnant horn toward the end of the mobile phase (Silva *et al.* 2005). The mobility phase lasts until around Day 15 of gestation when the conceptus settles and is fixed in one uterine horn by uterine contractions that lead to a tight envelopment by the endometrium (Ginther 1983a; Ginther 1983b). Fixation occurs on Day 16 to

17 and is facilitated by increased uterine tone and thickening of the uterine wall, allowing for increased folding of the endometrium (Ginther 1983b).

1.1.3.2 Implantation and formation of endometrial cups

Between Days 30 and 32, a subpopulation of trophoblast cells starts to differentiate into the invasive chorionic girdle, that is completed around Day 35 (Gerstenberg *et al.* 1999). Between Days 35 and 38 the chorionic girdle cells invade the maternal endometrium to form endometrial cups, that secrete equine chorionic gonadotrophin (**eCG**). This hormone is secreted into the maternal circulation and has GnRH-like properties, leading to the development of secondary follicles and corpora lutea (reviewed by Allen & Stewart 2001). The complete role of eCG in the mare is unknown, although it has been hypothesised to induce maternal immunotolerance to the conceptus. This has been disproved by *in vitro* studies (Lea & Bolton 1991), however it is hypothesised that the invading chorionic girdle cells prepare the uterine immunological environment for the microcotyledonary attachment of the chorionic placenta (Lunn *et al.* 1997). The invasion takes place with the girdle cells migrating through the uterine epithelium through to the stroma, involving a breakdown of surrounding extracellular matrix (**ECM**) and basement membrane (Vagnoni *et al.* 1995). The uterine environment has a role in regulating the timing of invasion by chorionic girdle cells (Vagnoni *et al.* 1996). It has been shown that the invasive properties of chorionic girdle trophoblast cells *in vitro* depends on the activity of MMPs as discussed in **1.3.6.4** (Vagnoni *et al.* 1995). The endometrial cups remain intact and functional until a maternal immune response results in their destruction and sloughing from the endometrium on Day 100 to 120.

1.1.3.3 Placentation, growth and development of placenta

True placentation does not take place until around Day 40 of gestation, when the non-invasive majority of the trophoblast cells start to interdigitate with the endometrium. Primary villi are seen by Day 60, that gradually give off secondary and tertiary branches to form the microcotyledonary structures characteristic of the mature equine placenta after Day 105 of gestation (Macdonald *et al.* 2000). The equine placenta continues to grow and develop through the latter half of gestation. This growth, involving increasing depth and branching of endometrial crypts with corresponding increase in villous digitation of foetal membranes, continues towards term (Macdonald & Fowden 1997; Macdonald *et al.* 2000). Placental thickness increases by 20% between 270 days of gestation and term (Macdonald *et al.* 2000).

1.1.3.4 Spatial limitations to placental function

Normal gross and microscopic development of the equine placenta is essential for prenatal growth and a successful pregnancy. It has been shown that placentation is delayed in mares with endometrial fibrosis (Bracher *et al.* 1996), probably due to the altered communication between cells in the foeto-maternal interface. Layers of fibrosis (see **1.1.6.2** and **1.1.6.3**) may render the endometrium rigid and inhibit its adaptation to foetal microvilli in the establishment of microcotyledons. By transferring embryos from pony mares to thoroughbred mares and vice versa, it has been shown that the size of a newborn foal depends on the volume of the uterine environment during gestation (Allen *et al.* 2002b). This principle is also demonstrated by the uterine development of equine twins where the

chorioallantois membranes of two foetuses compete for the endometrial surface, often leading to the death of one or both foetuses (Jeffcott & Whitwell 1973). In a retrospective study the incidence of twinning in the thoroughbred mare was 1-2%, and although this figure should be treated with caution due to the lack of observations of early aborted twins, it gives some idea of the prevalence (Jeffcott & Whitwell 1973). A more recent retrospective study of 1393 thoroughbred mares found that 6.8% of all pregnancies were twin pregnancies (Morris & Allen 2002). In a study of 509 cases of abortion and stillbirth in thoroughbred mares, 22% were found to be due to twinning, making this the largest single category of foetal mortality (Jeffcott & Whitwell 1973). The majority (73%) of foetal mortality occurred from 8 months of gestation to term, and the overall villous chorionic area of the placentae of both twins was only slightly greater than that for normal singleton foals (Jeffcott & Whitwell 1973). These results indicate that these losses are likely to be due to the inability of the uterus to sustain the foetuses as their nutritional needs become more demanding. In a study on twin gestation in a mixed-breed population of mares, draft horse mares had on average 1.4 live foals, crossbred mares had on average 1.3 live foals, whereas thoroughbred mares only had 0.5 live foals. It was concluded that the draft horse has greater uterine capacity to supply the needs of two foetuses during the second half of gestation (Vandeplassche *et al.* 1970). This further illustrates the importance of optimal functionality of the thoroughbred uterus in order to produce a viable foal.

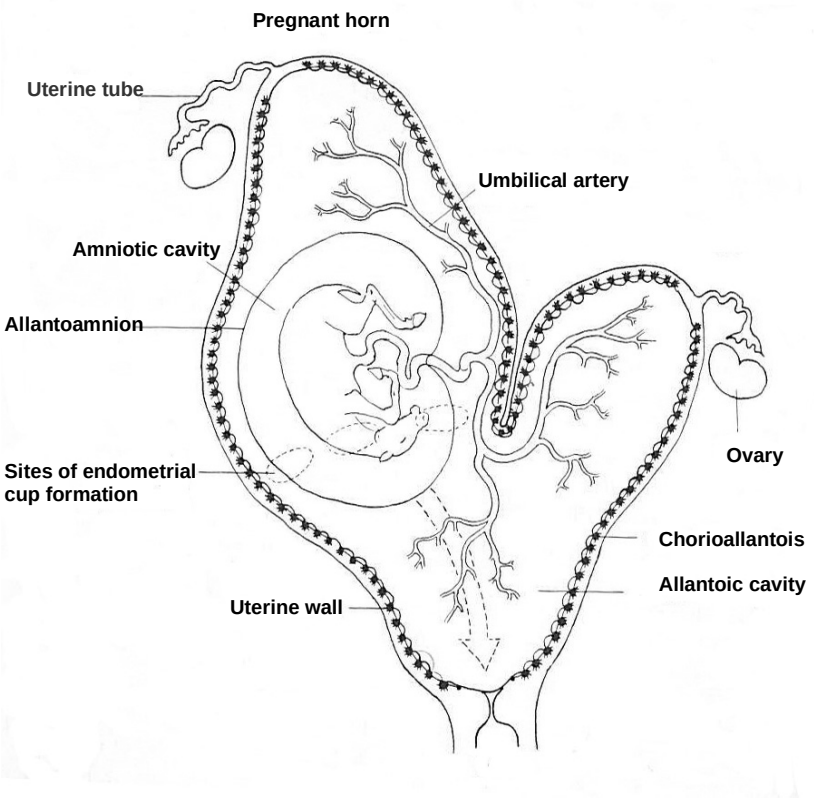


Figure 1.2 Schematic representation of the foetal membranes of the mare. Based on an aborted foetus at about five months. The dotted arrow shows the course normally followed by the allantoamnion at parturition (reproduced from Steven & Morriss 1975).

1.1.3.5 Foetal compartments and foetal fluids

Soon after the conceptus becomes fixed, the yolk sac starts regressing and the allantois grows larger. The allantois in the horse is unique, in that it expands over the dorsal surface of the amnion (**Figure 1.2**), so that its inner leaf is in contact with the outer surface of the amnion (forming the allantoamnion) and its outer leaf is apposed to the inner surface of the chorion (forming the chorioallantois; (Steven 1982). The allantoic cavity is larger than the amniotic cavity, thus containing more fluid; allantoic fluid measures 8-15 litres at term, whereas amniotic fluid is 3-5 litres at term (Arthur 1969). Allantoic fluid is composed of hypotonic urine and foetal

excretory products, whereas amniotic fluid consists of secretions from the foetal respiratory tract and oral cavity, fluid from the mare's circulation, as well as some amounts of foetal urine, depending on the developmental stage of the urogenital tract (Flood 1991).

1.1.3.6 Stages and duration of equine parturition

During the last 20-30 days of gestation, maternal plasma progesterone concentrations rise and then fall in the last 24 hours before foaling (Rossdale & Silver 1982; Haluska *et al.* 1987). It has been demonstrated that plasma progesterone concentrations are higher in pony mares than in thoroughbred mares during the last 20 days of gestation (Allen *et al.* 2002a). Stage I of parturition is defined by the onset of myometrial activity, and has been reported to commence 9-17 hours before parturition (Haluska *et al.* 1987) although it is difficult to determine by clinical signs in the mare. The fall in progesterone enables the initiation of myometrial activity in preparation for parturition. There is a reduction in myometrial activity two to four hours before parturition but the activity abruptly increases at the rupture of the chorioallantois (Haluska *et al.* 1987), marking the beginning of stage II. In a reported 21% of foalings the chorioallantois fails to rupture resulting in the appearance of the velvety red surface ('red bag') of the chorioallantoic membrane (Whitwell & Jeffcott 1975). This is certainly an overestimation as the diagnosis was based purely on the appearance of the placenta and not consistently associated with clinical observations. A 'red bag' delivery indicates a premature separation of the placenta, as the normal occurrence is for the chorioallantois to be expelled in an inverted state. When the chorioallantois ruptures the allantoic fluid escapes and the

transparent bluish amnion appears at the vulva. Stage II is extremely rapid, and takes on average only 20 minutes (10-60 minutes; (Rossdale & Ricketts 1980). The final stage (stage III) of parturition is the expulsion of the placenta, when the chorioallantois is freed from the uterine crypts starting cranially and proceeding in a caudal direction, resulting in the inversion of the membrane (Rossdale & Ricketts 1980).

1.1.4 Equine breeding methods

Mares are bred by two basic methods; natural service or artificial insemination (**AI**). Natural service involves either breeding mares under free-running pasture conditions or breeding them at hand, i.e. holding the mare while a stallion mounts her. The thoroughbred racing and breeding industry do not acknowledge foals born as a result of AI or embryo transfer, thus in order to be eligible for registration in the General Stud Book, a foal must be the result of a stallion's natural service of a mare (Weatherbys 2006). This requirement severely restricts the options available for breeding thoroughbred mares with fertility problems such as persistent breeding-induced endometritis (see **1.4.2** and **1.1.6.5**). Breeding by AI is performed by introducing stallion sperm via the cervix into the uterus. Semen used for AI can be used fresh, directly after collection from the stallion, or preserved by various methods. Preserving methods include refrigeration (chilling) and freezing, but not all stallions produce semen suitable for freezing as spermatozoa are extremely vulnerable to cold shock. To decrease the damage sustained by spermatozoa during chilling and freezing, the semen is mixed with an appropriate extender in a minimum dilution ration of 1:1 to 1:2 (semen to extender).

The most commonly used equine semen extenders are milk based, have an osmotic pressure compatible with spermatozoa and act to protect them against temperature changes (Pickett 1993). In order to freeze equine semen, glycerol or lactose are added to the extender to either decrease the freezing point of the solution or to dehydrate spermatozoa (Amann & Pickett 1987). Additionally, antibiotics can be added to the extender to greatly reduce the number of bacteria, that inevitably contaminate the seminal sample during collection (Pickett 1993). When breeding mares with fertility problems, such as persistent breeding-induced endometritis (see **1.4.2**) it is valuable to have a choice of breeding methods to minimize uterine inflammation (see **1.1.6.5**).

1.1.4.1 Reproductive efficiency depending on method of breeding

Breeding efficiency in horses is most accurately determined on the basis of pregnancy rate per oestrous cycle, although other definitions, such as pregnancy rate per season and foaling rate among mares bred during the previous season are more clinically applicable. In free-ranging Icelandic mares pregnancy and foaling rates per season are estimated 82% and 81% respectively (Hugason *et al.* 1985), and pregnancy and foaling rate per season has been estimated 88% and 83% respectively in registered thoroughbreds (Morris & Alen 2002). When using AI, lower pregnancy rates per oestrous cycle can be expected for frozen semen than for chilled semen. Many studies have been conducted on the effect of semen preservation methods on pregnancy rates, but due to varying research methods and definitions of fertility it is difficult to compare them. The pregnancy rate per cycle as diagnosed 30 days after AI with chilled extended semen stored for 70 hours and 80 hours was 77% and 57%

respectively (Heiskanen *et al.* 1994). A day 16-18 pregnancy rate of 33-50% was obtained using frozen semen in 401 mares (Sieme *et al.* 2003) and a pregnancy rate per cycle (day 13-35 after ovulation) of 45-48% was reported in a retrospective study of French warm-blood mares after AI with frozen semen (Vidament 2005).

1.1.4.2 Reduced mare fertility with ageing

Reproductive efficiency in mares declines with age, and it has been shown that mares aged over 14 years have lower Day 35 pregnancy rates per cycle than younger mares (Morris & Allen 2002). In a study on ponies, the Day 15 pregnancy rate for older mares was 38%, whereas younger mares had a rate of 100% (Carnevale & Ginther 1992). Among thoroughbred mares, live foal rate per season has been reported to decline from 75% in 4 year old mares to 48% in mares over 20 years (Jeffcott *et al.* 1982). In this study it was concluded that the failure to conceive was the most frequent cause of unsuccessful breeding in these mares. However, in recent years earlier pregnancy diagnosis in the mare by transrectal ultrasonography has been increasingly used, making it possible to distinguish embryo loss and fertilization failure. As a consequence, more recent studies have shown that embryo loss at an early stage, rather than conception failure, is the main cause of reproductive wastage seen in thoroughbred mares, with around 60% of all pregnancy losses occurring between Days 15 and 35 of gestation (Morris & Allen 2002). In ponies, embryo loss occurred from Day 24 to Day 30 in around 63% of old mares, but only one out of the 9 younger mares used in the study (11%) lost an embryo, occurring at Day 39 (Carnevale & Ginther 1992). In a study of 3740 mares of various breeds the abortion rate in mares under 15 years of age was 8%, but 17% in mares over 20 years

(Chevalier-Clément 1989). In this study a protective effect of parity against abortion was proposed.

1.1.4.3 Management of fertility problems

Absolute infertility in the mare is rare, but subfertility due to various causes is a common clinical problem, with endometritis (see **1.4.1** and **1.4.2**) ranked as the third most common clinical problem by 1,149 equine clinicians in the United States (Traub-Dargatz *et al.* 1991). Mares with a history of pregnancy loss can often carry a normal foal to term with intensive breeding management (Darenius *et al.* 1988), and foaling rate per season has been known to increase from 48% to 82% with intensive veterinary supervision (Doig *et al.* 1981). As well as employing breeding methods that minimize uterine contamination, intensive veterinary supervision includes the treatment of endometritis (see **1.4.1.5**) in the days following breeding. As concluded by Morris & Allen (2002) subfertile mares, susceptible to persistent endometritis (see **1.4.2**) can experience a successful pregnancy with intensive breeding management and uterine treatment. In a study on mares undergoing embryo transfer, it was found that older recipient mares tended to experience more embryonic death than younger ones although this was not statistically significant (Carnevale *et al.* 2000). This implies that, regardless of the source of the oocyte, there is an age-related increased risk of pregnancy loss. The age of donor mares did not make a statistical difference to 16 and 50 Day pregnancy rates in another study, although oocyte quality is known to be affected by age (Carnevale *et al.* 2005). In this study, however, there was less age difference between younger and older mares than in previous studies that might even out the differences between the groups. The

results from these studies indicate that the most important cause of early pregnancy loss in older mares is poor uterine environment.

1.1.5 Endometrial biopsy as a diagnostic aid

Kenney (1978) described the collection and interpretation of endometrial biopsies as an aid in the diagnosis of pregnancy loss in the mare, and postulated that serious fibrotic endometrial changes are the major cause of early embryonic or foetal death in the mare. This conclusion was made based on biopsies and reproductive data from 285 mares, demonstrating a foaling rate of 70-92% in mares without endometrial fibrosis and under 11% in mares with severe fibrotic changes (Kenney 1978).

A biopsy specimen of the endometrium usually includes an endometrial fold, occasionally with a portion of the inner circular layer of the myometrium. The specimen is collected by inserting a long alligator-jawed biopsy forceps through the cervix and into the uterine lumen as described previously (Ricketts 1975b). A wide array of histopathological changes can be observed in endometrial biopsies, including inflammation, fibrosis and cystic dilation of glands.

1.1.5.1 Histological description of endometritis in endometrial biopsies

The inflammation of the endometrium is referred to as endometritis and is defined as either acute or chronic by the dominating inflammatory cell type. Acute endometritis is characterised by neutrophils, primarily found in the stratum

compactum and migrating through the luminal epithelium to the uterine lumen, as they play a pivotal role in interacting with foreign material in the uterine lumen (see **1.4.4**). Chronic endometritis is characterised predominantly by lymphocytes and plasma cells, and less commonly by eosinophils and mast cells. Lymphocytes can be found in the stratum spongiosum as well as the stratum compactum, scattered or arranged in foci. Although primarily associated with chronic endometritis, T-lymphocytes have been shown to increase in numbers in the endometrium as early as 6 hours following AI (Tunón *et al.* 2000). Eosinophils are found in discrete and diffuse foci, mostly in the stratum compactum and have been reported more frequently in endometritis during oestrus, and in connection with the entrance of air into the vagina and uterus (Kenney 1978; Slusher *et al.* 1984). They have also been found to react to non-specific uterine irritation, such as repeated intrauterine infusion of iodine (Olsen *et al.* 1992). Plasma cells are found mostly in the upper stratum compactum but can be found anywhere in the lamina propria. They are the major producer of humoral antibody and are involved in the mucosal immune response, and the presence of high numbers of plasma cells indicates a continuing antigenic stimulation and a possible ongoing infection, as has been shown in experimental chronic bacterial infections (Ganjam *et al.* 1982; Acland & Kenney 1983;). Mast cells and macrophages can be seen scattered throughout the lamina propria but are most commonly seen in the stratum compactum (Kenney 1978).

1.1.5.2 Histological changes in the equine endometrium with ageing

Endometrial changes seen in older mares can be divided into two major complexes: chronic infiltrative endometritis (**CIE**) characterised by lymphocytic

infiltrations, and CDE characterised by glandular 'nests' and 'cysts', and periglandular and diffuse stromal fibrosis (Ricketts & Alonso 1991). Both complexes are commonly recognised in endometrial biopsies from subfertile mares. Cellular infiltrations seen in CIE are predominantly lymphocytic, with some eosinophils, plasma cells and neutrophils, indicating some superimposed acute inflammation (Kenney 1978; Carnevale & Ginther 1992). In one study of fertility in older mares during early gestation the amount of cellular infiltrations did not differ between mares that lost or maintained an embryo (Carnevale & Ginther 1992), and it has been shown that mare age does not affect the numbers of antigen presenting cells or T lymphocytes in the endometrium (Tunón *et al.* 1999b).

1.1.6 Endometrial fibrosis is due to the deposition of collagen in the stroma

Endometrial fibrosis is a prominent feature of the CDE complex and involves an abnormal deposition of collagen around the endometrial glands (periglandular) or in the endometrial stroma. Various types of collagen are constituents of the normal ECM, including the fibrillar collagens type I and III and the laminar collagen IV, that can be found in the basement membrane (Mansour *et al.* 2003). During the oestrous cycle, the stromal arrangement of collagen type I fibres varies. In oestrus, stratum spongiosum fibres are loosely arranged and stratum compactum fibres are densely arranged, whereas in dioestrus both stratum compactum and stratum spongiosum fibres are densely arranged (Walter *et al.* 2001). This difference is thought to be in part due to stromal oedema during oestrus, although this does not seem to influence the arrangement of collagen type III fibres, that are constantly present in a dense network throughout the oestrous cycle (Walter *et al.* 2001).

1.1.6.1 Cellular sources of endometrial collagen

The stromal cells in the lamina propria have the ability to deposit collagen as a response to local stimuli, such as chronic inflammation. Using picrosirius staining for collagen fibres, the amount of collagen was found to be increased in the endometrium of mares with endometrial fibrosis (Evans *et al.* 1998). In that study, it was concluded that the increased amount of collagen was due to fibrillar collagens (such as collagen types I and III). Other work, using immunohistochemistry, found that in mares with endometrial fibrosis the distribution of collagen types I and III did not differ from mares without fibrosis, but an increase was seen in collagen type IV content in mares with endometrial fibrosis when compared to mares without fibrotic changes (Walter *et al.* 2001).

1.1.6.2 Histological description of equine endometrial fibrosis

Kenney (1978) described the arrangement of fibrosis as periglandular or in association with the basement membrane of the luminal epithelium and pointed out that it was frequently found *without* inflammatory cells being present. Transmission electron microscopy demonstrated an increased density of collagen fibrils in the endometrial periglandular area accompanied by an increased number of fibroblasts (Evans *et al.* 1998). In severe periglandular fibrosis, several layers of activated fibroblasts (myofibroblasts) were surrounded by increased amounts of collagen IV, laminin and fibronectin (Walter *et al.* 2001). It was hypothesised that the contractile abilities of these cells would aid in the emptying of secretions from fibrotic glands,

although it is known from other organs that they play a central role in fibrosis (see **1.2.2.2**). Some myofibroblast differentiation was also seen around glands that had not developed fibrosis, indicating a possible early stage of endometrial fibrosis (Walter *et al.* 2001). It has been proposed that an inhibition of glandular emptying can lead to a cystic distension of the basal portion, resulting in periglandular fibrosis as a response to the glandular distension (Kenney 1978).

The first sign of fibrotic changes is the loss of randomization of fibroblasts in the stratum compactum and stratum spongiosum, most readily seen around glands (Walter *et al.* 2001). Periglandular fibrosis can involve individual gland ducts, individual branches of adjacent glands or ensheath all branches of one gland, forming a 'nest' of glandular ducts clustered together (**Figure 1.3d**). It appears that periglandular fibrosis seriously compromises the function of the involved gland, possibly by separating it from the underlying capillaries (Kenney 1978). This is known in other organs, such as interstitial fibrosis of the lung and kidney where the function of these organs is hampered due to the separation from the capillary network (see **1.2.2**). Cystic glands are often seen with or without the presence of fibrosis, and Kenney (1978) speculated that this was likely to be a reaction to the obstruction of flow of glandular secretions.

1.1.6.3 Quantification of equine endometrial fibrosis

For practical interpretation, Kenney devised an ordinal scale with three categories, based on qualitative and quantitative assessment of the presence and distribution of CIE/CDE lesions: I, slight; II, slight to moderate; III, severe (Kenney

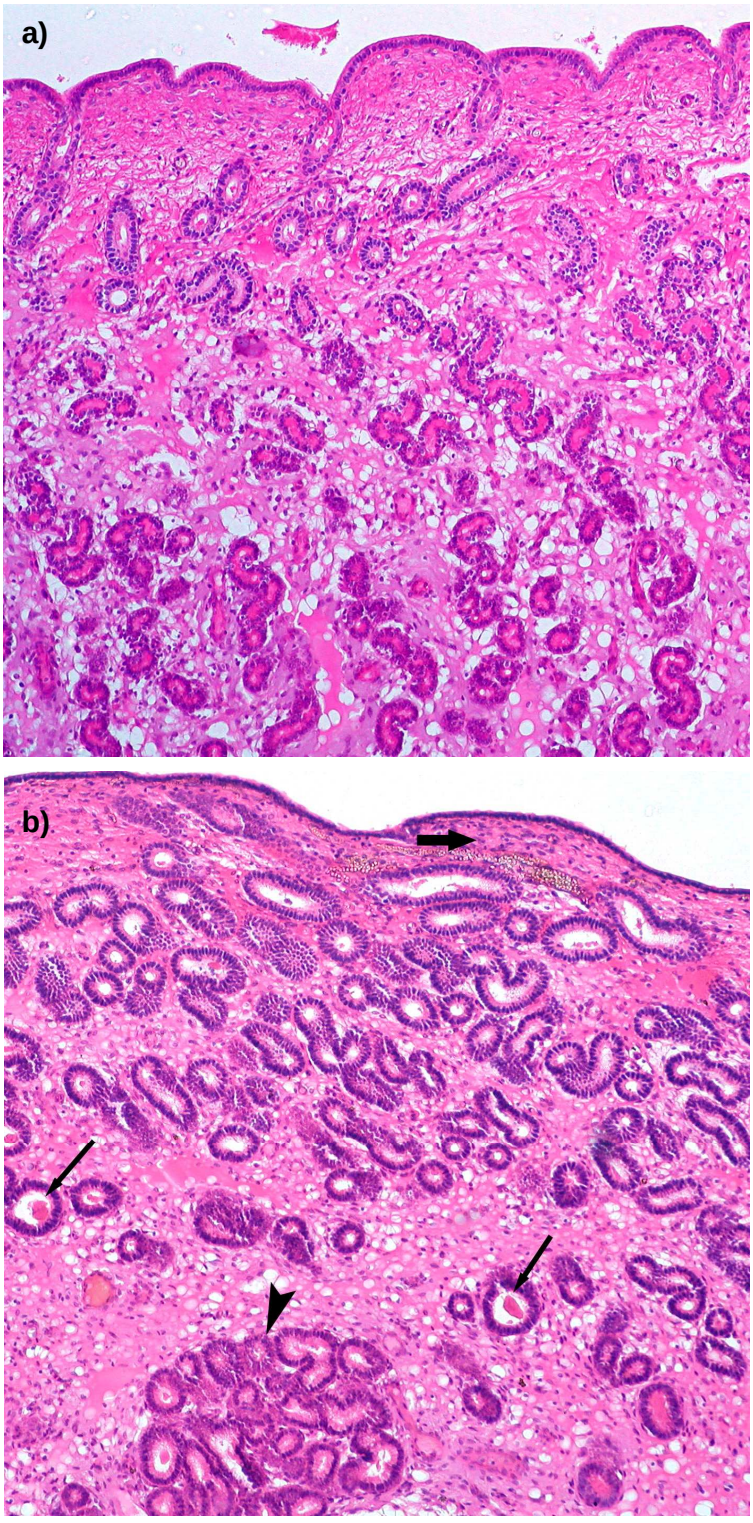
1978). A further subdivision of category II into IIA and IIB was later proposed, resulting in the four categories widely used today (Kenney & Doig 1986); **Table 1.1** and **Figure 1.3**).

Table 1.1 Categorization of mares based on histopathological findings in endometrial biopsies (Kenney & Doig 1986)

	Category I	**Category IIA**	**Category IIB**	**Category III**
Lymphocyte infiltration	Absent or very slight	Slight to moderate, diffuse	Widespread, diffuse	Widespread, concentrated
Lymphocytic foci	Absent or sparsely scattered	Scattered, frequent	Widespread, moderately severe	Widespread, severe
Fibrotic changes	Absent or sparsely scattered	Frequent, scattered around individual gland branches	Widespread, moderate to severe, evenly distributed	Widespread, periglandular and stromal fibrosis
Fibrotic glandular nests	Absent or very occasional	< 2 pr 5.5 mm linear field in four or more fields	2-4 fibrotic nests pr 5.5 mm in four or more fields	> 5 fibrotic nests pr 5.5 mm in four or more fields

This system is widely used in clinical diagnostics as it is a simple and quick way of assessing the reproductive health of a mare, but is highly subjective and requires experience and consideration of other factors, such as reproductive history in order to be reliable. When interpreting an endometrial biopsy for clinical purposes it is unavoidable to apply some subjective opinion. One study aimed to correlate age and parity with the degree of CDE and CIE indicated that CDE was a progressive condition that was inevitable within certain age limits, and was associated more with age than with parity. It was concluded that it was acceptable for mares aged 9 or older to have category IIA endometrium, category IIB was acceptable for mares over 13 years and category III was acceptable in mares 17 years and over (Ricketts & Alonso 1991). The study was carried out in a mixed-breed population and these conclusions have therefore been accepted for various horse breeds.

In mares with category III endometrium, a reduction in the number of epithelial cilia on glandular cells was observed (Ferreira-Dias *et al.* 1999). These cilia might have a role in the movement of glandular secretions toward the uterine lumen, most importantly during the first 35 days of gestation when the conceptus relies on the uterine glands for nutrition (Samuel *et al.* 1977). A pronounced increase in the presence of collagen fibres has been reported around uterine glands, under the basement membrane (**BM**) and scattered throughout the stroma in category III endometria (Ferreira-Dias *et al.* 1999). In some of these mares, a disruption to the arrangement of stromal and glandular cells was seen due to the bulk of collagen bundles. Reduced numbers of organelles in glandular epithelial cells as well as signs of cellular degeneration were more commonly seen in category III endometrium than in category I endometrium. The degeneration and reduced activity of these cells might be a result of a separation from the blood supply by the bulk of fibrotic lesions (Ferreira-Dias *et al.* 1999; Ferreira-Dias *et al.* 1994).


a)
b)

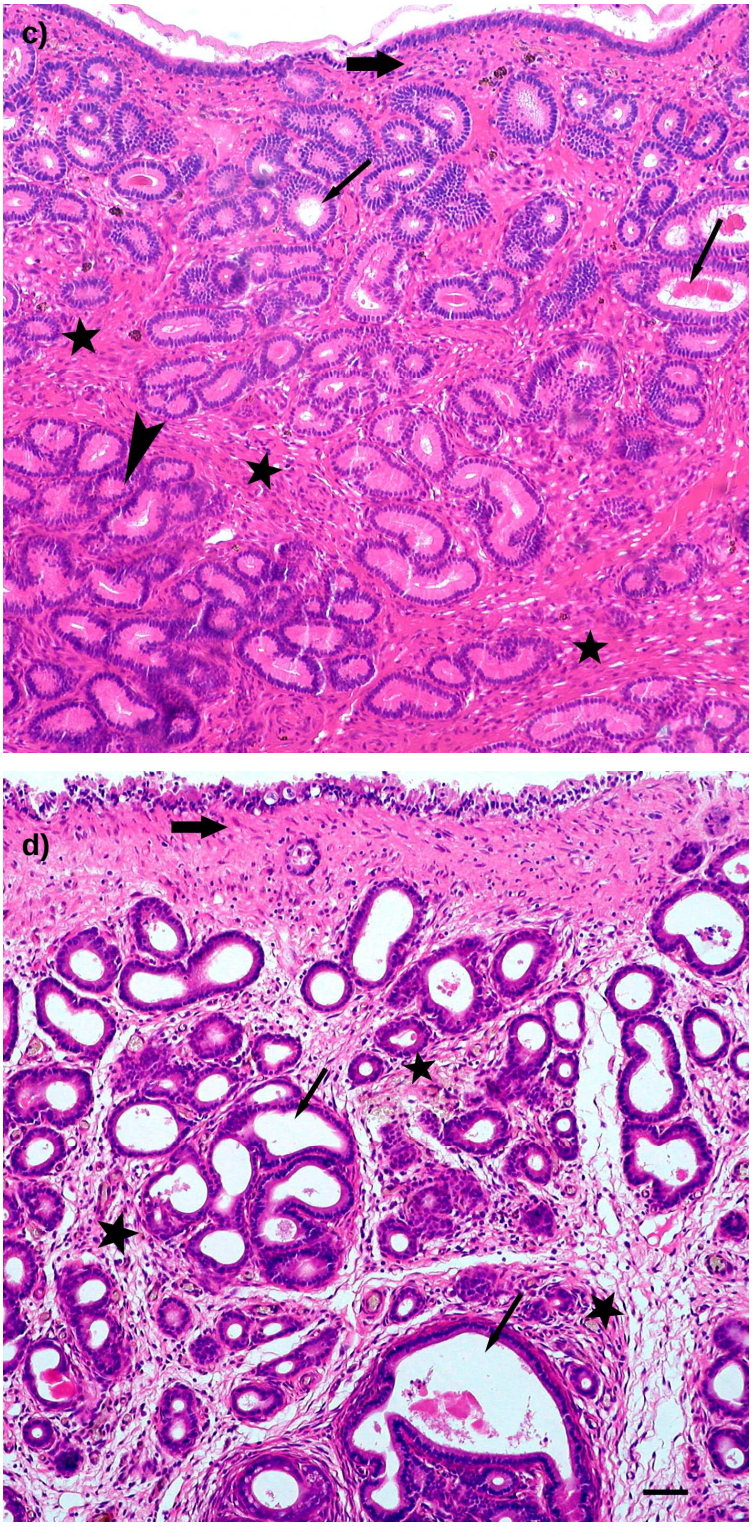

c)
d)

Figure 1.3 (see pages 27 and 28). Equine endometrial biopsies representative of the four categories of the Kenney classification system. Biopsies taken from four different mares in dioestrus. a) Category I endometrium from a 4 year old mare with no lymphocytic infiltrations and no fibrosis, and homogeneous distribution of glands in a loosely arranged stroma; b) category IIA endometrium from a 13 year old mare with slight lymphocytic infiltration (block arrow), slight distension of glands (arrows) and nonfibrotic glandular nest (arrowhead); c) category IIB endometrium from an 18 year old mare with a moderate diffuse lymphocytic infiltration (block arrow), slight distension of glands (arrows) and moderate to severe widespread periglandular fibrosis (stars) with fibrotic glandular nest (arrowhead); d) category III endometrium from a 17 year old mare with a mild scattered lymphocytic infiltration (block arrow), greatly distended glands (arrows), periglandular and diffuse stromal fibrosis (stars). Samples were fixed in Bouin's solution and stained with **H&E** (haematoxylin and eosin). Scale bar = 100µm.

1.1.6.4 Restricted function of equine fibrotic endometrial glands

Periglandular fibrosis affects glandular function in both non-pregnant and pregnant mares (Troedsson *et al.* 1993a; Bracher *et al.* 1996). The increased fibrotic changes and decreased density of endometrial glands were correlated with chronic inflammation or with age-related hormonal alterations (Carnevale & Ginther 1992). Indeed, the old mares had greater degree of intrauterine fluid accumulations (see **1.4.2**). Mares with intrauterine fluid accumulations are more likely to experience embryo-loss between Days 11 and 15 as a result of uterine-induced luteolysis due to the presence of inflammation, or inflammatory products causing direct damage to the embryo (Carnevale & Ginther 1992). In an attempt to elucidate the classification of susceptibility to persistent endometritis (see **1.4.2**) a study was carried out to investigate the correlation between histopathology and susceptibility to persistent endometritis. Category I mares were less likely and category III mares were more likely to be susceptible to persistent bacterial infection, however category IIA and IIB mares were inconsistently categorised as susceptible or resistant (Troedsson *et al.* 1993a).

It could therefore be concluded that histopathology and reproductive history do not provide sufficient information to classify a mare as potentially susceptible to persistent endometritis. In a standardised experimental setting, it is therefore valuable to challenge-expose mares to an intrauterine bacterial inoculation to determine their resistance or susceptibility to persistent endometritis (see **1.4.2**). In a clinical situation, the most effective method is combining reproductive history, clinical signs, and results of endometrial biopsy and cytological assessment (Troedsson *et al.* 1993a).

1.1.6.5 Studies on the aetiology of equine endometrial fibrosis

In a study of 700 subfertile mares, endometrial fibrosis was found in endometrial biopsies from 88% of mares and increasing severity of fibrosis was positively correlated to mare age, number of years barren and incidence of foetal loss in the three preceding years (Doig *et al.* 1981). Half of these mares were bred under intensive veterinary supervision, using artificial insemination, and the other half were bred under less stringent management systems, mostly natural service and often without direct veterinary supervision. In the intensively managed mares the degree of fibrosis and years barren did not influence the foaling rate until in category IIB and two or more years barren. In the less intensively managed mares the foaling rate was strongly influenced by the degree of fibrosis and number of years barren, where foaling percentages dropped dramatically at category IIA and barren two years or more. It was therefore shown that optimal breeding management can overcome to a certain degree the detrimental effects of fibrosis on foaling rates. In this study, average age increased with advancing category, however there was a wide range of

age in each category (Doig *et al.* 1981). It has been speculated that not only ageing, but also increased parity increases age-related subfertility due to endometrial degeneration. In another study there were no differences in the incidence of pregnancy loss per season between maiden mares (15%) and barren (12%) or foaling (19%) mares, indicating that age, more than parity, influences the condition of the endometrium (Morris & Allen 2002).

To conclude, equine endometrial fibrosis is the result of the deposition of excessive amounts of collagen in the endometrial periglandular or stromal areas and appears to be associated with an increased number of myofibroblasts. This is consistent with fibrosis pathology in other organ systems, such as the liver, that has been extensively researched (reviewed by Iredale 1997). Therefore, it is valuable to study the pathogenesis of fibrosis in other tissues in order to elucidate the mechanisms behind endometrial fibrosis.

1.2 General tissue remodelling and fibrosis

Fibrosis can be defined as an excessive deposition of collagen that results in a destruction of normal tissue architecture and a compromise in tissue function. However, the deposition of collagen is not always a pathological process, as it is important in the healing response following injury. Inflammation is the most common means by which tissues become injured and is closely intertwined with the responses that constitute healing and repair. Repair is aimed at reconstituting tissue structural and functional integrity via either parenchymal regeneration or by filling the defect with less specialised fibroblastic connective tissue. Fibroblasts are

essential to wound repair as they are able to proliferate locally and to synthesize and lay down ECM components such as collagen to restore functional and structural integrity (reviewed by Lorena *et al.* 2002). They are ubiquitous connective tissue cells and the most widespread of the mesenchymal cells, found in substantial numbers in the ECM of most tissues in the body.

1.2.1 Normal tissue repair following injury

Tissue injury can be part of physiological processes, such as menstruation in the human, although the underlying mechanisms are the same as in pathological processes. During tissue healing there is rapid synthesis and degradation of connective tissue proteins (especially collagens), a process referred to as *remodelling* (reviewed by Mutsaers *et al.* 1997). Collagenolytic activity assists in allowing wound healing to enter the proliferative phase by freeing cells for migration and proliferation. This event can in turn be shown to be associated with a burst in collagen synthetic activity by activated fibroblasts, referred to as *scar formation* (reviewed by Mutsaers *et al.* 1997). In healing wounds, among the factors activating collagen synthesis and contractile properties by fibroblasts are a range of high molecular weight proteins in wound fluid (Schäffer *et al.* 1997).

Activated fibroblasts develop a fibrillar cytoplasmic system similar to the fibrils of smooth muscle cells and are therefore called myofibroblasts (reviewed by Lorena *et al.* 2002). As a consequence they have the ability to contract, thus shrinking the scar tissue being formed. The biochemical composition of ECM depends partly on the time elapsed since the injury occurred, with fibronectin a prominent component of

young healing wounds, whereas collagen is more apparent in older wounds. Fibronectin acts as an attractant and adhesive for inflammatory cells at the site of injury and as a primitive template for the collagens gradually synthesised by fibroblasts (Greiling & Clark 1997). The wound healing tissue undergoing active remodelling is referred to as granulation tissue, and this is gradually replaced by collagen types I and III (reviewed by Carlson & Longaker 2004). Collagen III is the major form in early lesions, and type I predominates in advanced lesions (reviewed by Franklin 1997). The BM can be reformed in sequential stages with deposition of type IV collagen (Chaudhuri & Karasek 2006).

The final stage of healing involves ECM maturation and removal by the breaking up of collagen synthesised during the proliferation phase of inflammation, in a *resolution* of the scar (reviewed by Mutsaers *et al.* 1997). The degradation of collagen is controlled by various collagenase enzymes, such as the MMPs from inflammatory cells as well as fibroblasts and other resident mesenchymal cells. From the above it is therefore clear that during the first stage of wound healing, collagen synthesis is in excess of degradation, whereas during the last stage, degradation has the upper hand. Both wound healing and pathological fibrosis involve collagen synthesis and degradation, but the main difference lies in the fact that wound healing is a self-limiting process, whereas fibrosis is progressive and does not reach the resolution stage of collagen degradation, resulting in the deposition of scar tissue.

1.2.2 Pathological deposition of collagen resulting in fibrosis

Pathological fibrosis occurs as a consequence of defects in tissue remodelling following injury in various organs, such as liver (reviewed by Iredale 1997), kidney (reviewed by el Nahas *et al.* 1997), oral mucosa (Yoshimura *et al.* 2005) and lung (reviewed by Marshall *et al.* 1997). The resolution stage that normally follows tissue repair is not reached, resulting in uncontrolled fibroblast activity with excessive accumulation of ECM components, especially collagens type I and III, but also type IV (Milani *et al.* 1990). The thickened interstitium with increased collagen content compromises the function of the organ by physically separating cells and structures that need to be apposed for their biological function. This is seen in pulmonary fibrosis, where the normally thin BM shared between the epithelial and endothelial cells is thickened and leads to a reduction in gas transfer (reviewed by Marshall *et al.* 1997). The same is true for the kidney, where the glomerulus is separated from the renal tubules, thus reducing the filtering capacity of the organ (reviewed by el Nahas *et al.* 1997).

1.2.2.1 Pathogenesis of fibrosis in the liver

Liver fibrosis represents the wound healing response to chronic hepatic insult and can be used as a model for the scarring process in other organs (reviewed by Iredale 1997). Most of the molecular and cellular signals in liver injury are centred on the hepatic stellate cell (**HSC**), that as a response to injury is transformed into a myofibroblast-type cell (reviewed by Marra 1999). Mesenchymal cells in other organs also take on myofibroblast-like phenotypes, such as the mesangial cells in the kidney (reviewed by el Nahas *et al.* 1997) and adventitial fibroblasts in the lung

(Adler *et al.* 1989). Myofibroblast-like cells have also been associated with equine endometrial fibrosis (Walter *et al.* 2001) as described in **1.1.6.2**. These and other cells have the ability to break down and synthesise ECM components, and are therefore central in both matrix degradation

and matrix synthesis.

1.2.2.2 The role of myofibroblast-like cells in organ fibrosis

The HSC are mesenchymal cells, that in the normal liver reside in a BM-like matrix between the hepatocytes and hepatic endothelium (reviewed by Marra 1999). As a result of injury they are 'activated' to express α-smooth muscle actin, proliferate and secrete matrix proteins, especially collagens I and III (Milani *et al.* 1990). In the kidney, the secretion by activated 'mesangioblasts' results in the presence of these collagens in the glomerulus, where the only collagen normally present is collagen IV in the BM (reviewed by el Nahas *et al.* 1997). It is generally accepted that myofibroblast-like cells are central to the fibrotic process as mediators of matrix synthesis, assimilation and degradation (reviewed by Mutsaers *et al.* 1997; reviewed by Wu & Zern 2000; reviewed by Phan 2002). Among the fibroblast activating factors is transforming growth factor (**TGF**)-β from resident macrophages (Kupffer cells) and HSC autocrine secretion in the liver (Sanderson *et al.* 1995). TGF-β1 is a key mediator of human fibrogenesis (reviewed by Gressner *et al.* 2002). Additionally, as a consequence of Kupffer cell activation, tumour necrosis factor (**TNF**)-α production is induced, in turn increasing neutrophil infiltration (reviewed by Bataller & Brenner 2005). Neutrophils, among other cells contain MMPs,

important ECM modulating and signalling factors, that are expressed in cultured activated HSC as well as Kupffer cells (reviewed by Iredale 1997).

1.2.2.3 Genetic and environmental predisposition to organ fibrosis

A genetic predisposition has been suggested to play a role in the development of pulmonary fibrosis, as only a small group of patients will develop the condition after receiving similar doses of the same drug (Tisdale *et al.* 1995). In a familial form of pulmonary fibrosis, provoked by a chronic state of macrophage activation and neutrophil recruitment, it has been suggested that individuals heterozygous for a particular allele have subclinical pulmonary disease, but never develop pulmonary fibrosis as do individuals that are homozygous (Bitterman *et al.* 1986). Genetic and environmental factors influencing liver fibrosis have also been identified, possibly explaining a broad spectrum of fibrosis severity in response to the same aetiological agent (reviewed by Bataller *et al.* 2003).

1.2.2.4 Delaying fibrosis by inducing scar resolution

It has been shown that the fibrotic process in liver, lung and kidney can be retarded or arrested by suppressing or eliminating injurious agents (reviewed by Franklin 1997). The failure of the disease to resolve is correlated with the persistence of myofibroblasts. In normal wound healing, the number of myofibroblasts gradually decreases, but in progressive fibrosis they persist and can be found in various stages of human lung fibrosis. Myofibroblast disappearance normally occurs by apoptosis, possibly due to the loss of TGF-β signalling (reviewed by Phan 2002). It has been shown that TGF-β increases matrix and collagen

synthesis, both directly, and by inhibiting MMP production and increasing the expression of tissue inhibitors of MMPs (**TIMP**) in the human endometrium (reviewed by Godkin & Doré 1998), although it has been reported as an activating factor for MMP-2 and -9 in inflammatory conditions (see **1.3.5**). Furthermore, TGF-β has been shown to have a biphasic effect on the proliferation of lung fibroblasts, stimulating at low concentrations and inhibiting at higher concentrations (McAnulty *et al.* 1997). In a study investigating equine endometrial mRNA expression of TGF-β1 and TNF-α, no association was made between the presence of fibrosis and the expression of these factors, and no differences were found between old and young mares (Cadario *et al.* 2002). The study did not investigate the expression of TGF-β1 protein and TNF-α receptors, and so would not have detected differences in the regulation at the protein or receptor level.

Tissue repair revolves around the activated fibroblast, or myofibroblast, that has the ability to synthesise collagens as well as the MMPs that degrade these collagens. Inflammatory factors, such as TGF-β from macrophages induce an activation of tissue fibroblasts, and this activation induces collagen synthesis. When TGF-β secretion diminishes, the number of myofibroblasts is reduced, and the collagenolytic activity of MMPs is induced, to restore tissue functionality. During chronic injury, such as occurs in mares with persistent endometritis (see **1.4.2**) macrophages or other inflammatory cells may constantly stimulate myofibroblasts, and inhibit collagenolysis and therefore collagen deposition occurs without resolution.

1.3 The matrix metalloproteinases

The MMPs are important in the normal tissue remodelling and play an important role in scar resolution by degrading collagen deposited by myofibroblast-like cells in the stroma. They are enzymes with a wide range of functions in the ECM, including cell-cell and cell-ECM interactions. Cells rely on the ECM not only for structural support, but also for signalling to influence cell shape, movement, growth and differentiation (reviewed by Lukashev & Werb 1998). By altering the composition of the ECM, the MMPs are important in altering matrix-derived signals. The result of MMP activity can be a degradation of the matrix, leading to the apoptosis of cells due to the loss of anchorage, as is seen during the involution of the mammary gland (Alexander *et al.* 1996). Other physiological processes involving the MMPs are embryo implantation and placentation that require considerable tissue remodelling of both endometrium and foetal membranes. During human pregnancy, the endometrium is invaded by trophoblast cells, that have been shown to secrete MMPs (Polette *et al.* 1994) at their most invasive stage during the first trimester as detailed in **1.3.6**. This is also true for the invasive subpopulation of trophoblast cells involved in the formation of equine endometrial cups (see **1.1.3.2**).

These physiological processes require the MMPs to be strictly regulated, and without this regulation they could indiscriminately degrade cell matrices and lead to widespread organ malfunction. Indeed, many pathological processes involve MMP regulation and therefore these enzymes have been extensively researched in order to elucidate the pathogenesis of these conditions. The invasive properties and uncontrolled growth of cancerous cells involve the activities of MMPs (reviewed by

Björklund & Koivunen 2005). During inflammation, MMPs effect the migration of leukocytes through tissue barriers, as well as modifying cytokines and chemokines (reviewed by Hu *et al.* 2007). In the horse, MMPs are induced in chronic obstructive pulmonary disease (Raulo 2001), as well as in inflammatory joint disease (Clegg & Carter 1999). As already mentioned in **1.2.1** and **1.2.2**, MMPs are important in the repair process and tissue fibrosis.

1.3.1 The matrix metalloproteinase family

The matrix metalloproteinases receive their name from their dependence on metal ions for catalytic activity, their potency in degrading structural proteins of the ECM and their specific structure, distinguishing them from other related metalloproteinases (reviewed by Sternlicht & Werb 2001). Two systems of nomenclature are in use simultaneously, one based on their common names and the other a sequential numeric system reserved for the vertebrate MMPs (reviewed by Sternlicht & Werb 2001).

1.3.1.1 Structure of matrix metalloproteinases

The vertebrate MMPs are a family of over 25 members, defined by their distinct but often overlapping substrate specificity (reviewed by Sternlicht & Werb 2001). Essentially, all MMPs have the same three-domain structure as illustrated in **Figure 1.4**. These are: (i) a hydrophobic N-terminal *signal peptide sequence* that is connected to the *propeptide sequence* that maintains the latent form of the MMP until removed upon activation; (ii) a *catalytic domain* containing the *zinc-binding site* that is chelated by conserved cysteine in the propeptide domain to maintain the

inactive form of the enzyme. This process of inhibition can be utilised for identification purposes *in vitro* (see **2.4.3.1**) by adding chemical chelating agents, such as ethylenediaminetetraacetic acid (**EDTA**) and 1,10-phenanthroline (Auld 1995); and (iii) a C-terminal (all MMPs except MMP-7 and -26) *hemopexin domain*, that confers much of the substrate specificity to the MMPs and binds to TIMPs, resulting in the inhibition of the MMP.

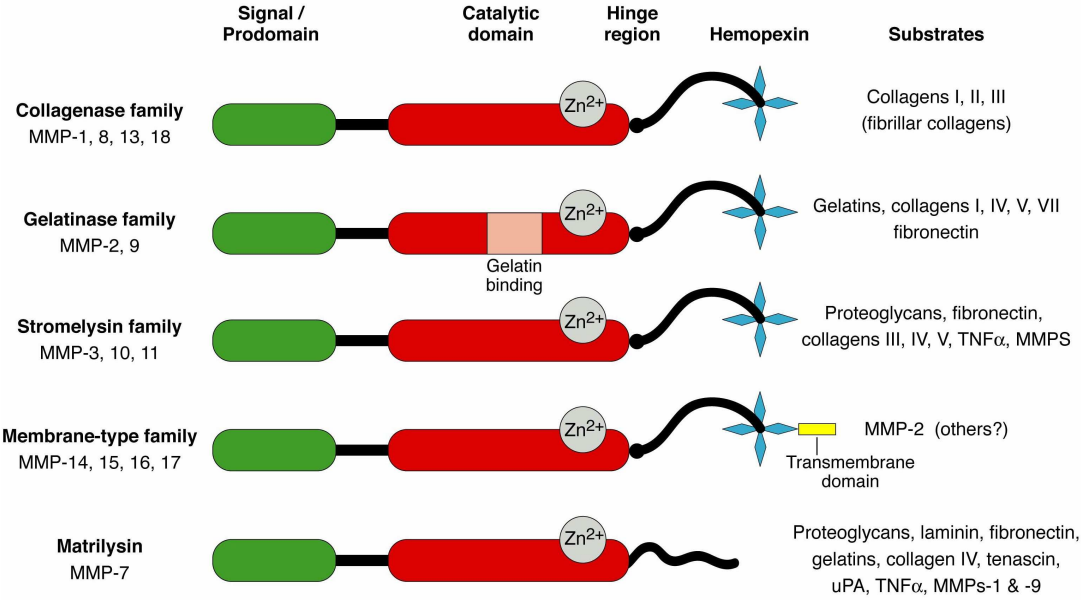


Figure 1.4 Domain structure of the matrix metalloproteinases (modified from Alexander 2002).

1.3.1.2 Regulation of matrix metalloproteinases occurs on many levels

The extreme potency of MMPs to degrade most components of the ECM necessitates rigorous regulation at several levels, as is underlined by the domain structures detailed in **1.3.1.1**. The regulation of MMPs occurs: (i) at transcriptional level by activation of the gene (signal peptide); (ii) by activation of the latent proenzyme by cleaving the propeptide sequence; and (iii) by extracellular inhibition

by the specific TIMPs or by more general protease inhibitors. TIMPs act by stabilising the proenzyme and by inhibiting the active species. The binding to an active MMP is reversible, and the separated TIMP retains its inhibitory activity (reviewed by Sternlicht & Werb 2001).

1.3.1.3 Nomenclature of matrix metalloproteinases

Each MMP is referred to by a common name or according to a sequential numeric nomenclature. The proenzyme is referred to as the latent MMP or proMMP, followed by its sequential number whereas the activated enzyme is referred to as the active MMP, followed by the sequential number. The MMPs can be divided into the following five subgroups by their structure and substrate specificity: 1) *collagenases* cleave interstitial collagens I, II and III; 2) *gelatinases* cleave gelatin, collagen IV, fibronectin among others, as detailed in **1.3.2**; 3) *stromelysins* are structurally similar to collagenases, but lack the ability to cleave interstitial collagens; 4) membrane-type MMPs (**MT-MMPs**) are cell membrane-associated proteins with the ability to activate MMP-2 and cleave collagens; 5) matrilysins lack the hemopexin domain and process cell surface molecules, as well as digesting ECM components (reviewed by Nagase *et al.* 2006). This study concentrated on the gelatinases and they will therefore be described in more detail.

1.3.2 The gelatinases MMP-2 and MMP-9

In addition to the above described generic structure, the gelatinases MMP-2 and MMP-9 have an addition of three collagen-binding type II repeats of fibronectin within their catalytic domain, making for their ability to bind and cleave collagen and

gelatin (denatured collagen). Their substrates include ECM proteins, such as collagens, fibrinogen, cell surface receptors and chemotactic molecules among others, and they are the only MMPs able to degrade collagen IV, that is only found in BM (reviewed by Björklund & Koivunen 2005). The binding to native collagens and gelatin occurs primarily via the collagen binding site (Chen *et al.* 2003), although gelatin also binds to the C-terminal (Collier *et al.* 2001; Roeb *et al.* 2002).

Although the gelatinases have many similarities, there are significant differences in the regulation of expression, glycosylation, proenzyme activation and substrate selectivity. Typically, MMP-2 is constitutively expressed with only minor up- or downregulation under various conditions (reviewed by Birkedal-Hansen *et al.* 1993). Conversely, MMP-9 expression is highly inducible and depends on the control of growth factors, chemokines and other stimulatory signals (Hipps *et al.* 1991). This difference is also due to structural differences, in this case in the promoter region (reviewed by Westermarck & Kähäri 1999).

1.3.2.1 Expression and activity of gelatinase A/MMP-2

MMP-2 (Gelatinase A/type IV gelatinase) is secreted in a 72kDa nonglycosylated latent form and is cleaved into 62 to 59kDa active forms in proteolytic activation (reviewed by Birkedal-Hansen *et al.* 1993). MMP-2 is abundantly expressed by many cell types, such as fibroblasts and other resident connective tissue cells, for example in equine articular cartilage (Clegg *et al.* 1997a). It is notable that MMP-2 is able to degrade native type I collagen, most likely due to a subsite in the catalytic site, that differs from MMP-9 (Chen *et al.* 2003). MMP-2 is

unique in terms of regulation, as it is often constitutively expressed and controlled through a unique mechanism of enzyme activation, involving MMP-14 (MT1-MMP) and TIMP-2 (Strongin *et al.* 1995).

1.3.2.2 Expression and activity of gelatinase B/MMP-9

MMP-9 (Gelatinase B/type IV gelatinase) is secreted in a 92kDa glycosylated latent form and is converted into 82 to 68kDa active forms when activated (reviewed by Van den Steen *et al.* 2002; reviewed by Björklund & Koivunen 2005). In addition to these forms, it exists in plasma as a dimer of 210 kDa and in neutrophil granules as a lipocalin complex of 125-130 kDa (Olson *et al.* 2000). MMP-9 is secreted by cultured equine neutrophils and monocytes (Clegg *et al.* 1997a), and is stored in cytoplasmic granules, that are delivered to the cell surface and excreted to the extracellular space (Borregaard 1997). It has been reported that human lymphocytes regulate the synthesis of MMP-9 by neutrophils, monocytes and macrophages (Lacraz *et al.* 1995; Seki *et al.* 1995).

1.3.3 Activation of gelatinases

The activation of MMPs requires cleavage or disruption of the propeptide. The activation process involves the critical "cysteine switch", the disruption of the conserved cysteine sequence in the prodomain (Van Wart & Birkedal-Hansen 1990). The cysteine sequence serves to block the entry of a catalytically essential water molecule to the active site, and the various activation mechanisms of MMPs all disrupt this interaction by removing the prodomain or by modifying the cysteine sequence within the prodomain. This allows a water molecule to bind to the catalytic

site and results in the formation of an active catalytic site (Van Wart & Birkedal-Hansen 1990).

The environment in which the MMPs are activated requires signals from surrounding cells and ECM. During inflammation, numerous cytokines and chemokines are secreted and act as signalling molecules to activate MMP secretion and activation, either directly or by attracting inflammatory cells that secrete MMPs. Among cytokines and growth factors with the ability to activate MMP-9 expression are epidermal growth factor (**EGF**), interleukin (**IL**)-1, TNF-α, and TGF-α and β (reviewed by Van den Steen *et al.* 2002). *In vitro* studies have demonstrated the ability of IL-1β to activate MMP-2 secreted by equine chondrocytes and fibroblasts (Clegg & Carter 1999).

Unlike most MMPs, MMP-2 is not activated by free serine proteases in the extracellular environment, but is activated at the cell surface through a unique multistep pathway involving MT1-MMP and TIMP-2 as already mentioned (Strongin *et al.* 1995). However, as described in **1.3.4.2,** the N-terminal domain of TIMP-2 is an MMP inhibitor, and at higher levels, TIMP-2 inhibits the activation of MMP-2 by blocking the removal of its prodomain (Strongin *et al.* 1995).

1.3.4 Inhibition of gelatinases

The inhibitory mechanisms of MMPs have been researched extensively in the search for therapeutic targets for diseases such as cancer and pathological fibrosis in an effort to readdress the MMP:TIMP balance (Baker *et al.* 2002). Inhibitors of

MMPs include naturally occurring endogenous compounds, such as α2-macroglobulin (a plasma proteinase inhibitor) and the TIMPs, as well as exogenous chemical inhibitors.

1.3.4.1 Tissue inhibitors of matrix metalloproteinases

The tissue inhibitors of metalloproteinases are a family of at least four 20 to 29-kDa secreted cysteine rich proteins that reversibly inhibit the MMPs in a 1:1 stoichiometric fashion (reviewed by Sternlicht & Werb 2001). TIMPs 1, 2, 3 and 4 vary in tissue specificity and their ability to inhibit various MMPs. TIMP-1 inhibits MMP-9 with high affinity, whereas TIMP-2 at high concentrations inhibits MMP-2 (Olson *et al.* 1997). The inhibitory activity of the TIMPs resides in the N-terminal, although the C-terminal mediates interactions with the catalytic domains of some MMPs, and the hemopexin domains of MMP-2 and MMP-9 (reviewed by Baker *et al*. 2002). All four TIMPs are abundant in female reproductive tissues (reviewed by Fata *et al.* 2000). The activities of the TIMPs include roles other than inhibiting MMP activity, and TIMP-1 and -2 have been shown to have a stimulating effect on the growth of some cell types, that in the case of TIMP-2 includes fibroblast proliferation (Corcoran & Stetler-Stevenson 1995).

1.3.5 The role of MMPs and TIMPs in fibrosis

In liver fibrosis, the MMPs are secreted by HSC and Kupffer cells (see **1.2.2.2**). These cells also synthesize TGF-β, a factor that possesses powerful profibrogenic activity, and is a pivotal cytokine in the fibrotic process and the regulation of MMPs, as discussed in **1.2.2.4**. Tubulointerstitial fibrosis of the kidney

is associated with a decrease in MMPs and an increase in TIMPs (Jones *et al.* 1995; Schaefer *et al.* 1996). Tubulointerstitial fibrosis as seen in diabetic nephropathy in rats is associated with decreased activity of MMP-9 and -2 in renal cortex, and decreased immunostaining of both MMPs in renal tubules, as well as an increase in the activity of TIMP-1 and -2 in renal cortex (Mankhey *et al.* 2007). In concert with those changes, an increase was seen in the protein expression of collagen type I and α type IV in a Western blot analysis on renal cortex (Mankhey *et al.* 2007). In this study researchers also identified a protective role of oestradiol, as no increase was found in collagen, MMP and TIMP expression in diabetic rats receiving oestradiol (Mankhey *et al.* 2007).

Gene expression of various MMPs is induced in cultured fibroblasts by EGF, IL-1, IL-6 and TNF-α (reviewed by Mauch *et al.* 1994). MMP-9 is induced by TGF-β1, whereas MMP-2 is only moderately induced (Overall *et al.* 1991; Salo *et al.* 1991). TIMP-2 is a more effective inhibitor of the gelatinases than TIMP-1 (Goldberg *et al.* 1989). The MMPs have been suggested to be more relevant therapeutic targets than the TIMPs, as mice deficient in the TIMP-1 and TIMP-2 genes do not show differences in hepatic fibrosis (Vaillant *et al.* 2001), and TIMP-1-null mice do not have an altered tendency to develop renal fibrosis (Kim *et al.* 2001).

1.3.6 Previous studies on MMPs in female reproductive tissues

The gelatinases have been extensively researched in female reproductive tissues and found to be important in growth and development of the conceptus and placenta, parturition and uterine involution, as well as in remodelling of the

menstruating endometrium and cycling ovaries (reviewed by Hulboy *et al.* 1997; reviewed by Salamonsen & Woolley 1999; reviewed by Curry, Jr. & Osteen 2003). Much of the research in the non-pregnant female has been focused on the human and mouse reproductive tracts, although studies have been carried out on MMP activity in equine ovarian follicles. Throughout equine follicular development, latent MMP-2 is secreted into follicular fluid in similar amounts and latent MMP-9 is also present although in smaller amounts (Riley *et al.* 2001). The TIMPs are all secreted in some amount into the follicular fluid. MMPs and TIMPs were localised in granulosa, theca and stromal cells (Riley *et al.* 2001). Immediately prior to ovulation, in the presence of a decreasing oestrogen:progesterone ratio in plasma, the activity of latent MMP-9 decreased significantly whereas latent MMP-2 activity did not change (Riley *et al.* 2004).

1.3.6.1 Regulation of endometrial MMPs by reproductive hormones

Progesterone is a key inhibitor of MMP-2 and -9 expression and activation in cultured human endometrial cells, most likely due to receptor mediated regulation (reviewed by Henriet *et al.* 2002). Conversely, the secretion of TIMP-1, -2 and -3 is induced by increased progesterone concentration. Oestrogen seems to have no effect on TIMP secretion, and its mild inducing effect on some of the MMPs depends on the concentrations of progesterone. The control of MMP expression in response to progesterone and oestrogen is controlled by cytokines, especially IL-1α and TGF-β (reviewed by Henriet *et al.* 2002).

1.3.6.2 MMPs and tissue remodelling in the endometrium

The female reproductive system has a unique physiological role involving repeated remodelling without scarring or loss of function. In the human endometrium, MMPs are vital in tissue remodelling and repair throughout the menstrual cycle (reviewed by Salamonsen & Woolley 1999). Expression of MMP-9 occurs among stromal, vascular and infiltrating cells (reviewed by Curry, Jr. & Osteen 2003), with subpopulations of MMP-9 immunopositive macrophages, eosinophils and neutrophils, their immunopositivity depending on the differentiation and activation status of the cell (reviewed by Salamonsen & Woolley 1999). Throughout the cycle, MMP-2 is constitutively expressed in stromal cells (Rodgers *et al.* 1994), whereas very limited MMP-9 expression is observed in glandular epithelium, neutrophils and macrophages (reviewed by Fata *et al.* 2000). TIMP-1 mRNA is expressed in endometrial stroma and epithelium throughout all stages of the menstrual cycle, most intensely during menstruation (Rodgers *et al.* 1994), whereas TIMP-2 remains high with little cyclical fluctuations. Both have been immunolocalised to the endometrial stroma, and strongly to vascular structures, indicating a role for these TIMPs in maintaining vascular integrity (reviewed by Fata *et al.* 2000). TIMP-3 has also been found in all tissue compartments of the human endometrium throughout the menstrual cycle, whereas the expression of TIMP-4 is less known (reviewed by Curry, Jr. & Osteen 2003).

1.3.6.3 MMPs and remodelling during endometrial inflammation

Human menstruation is a physiological, and not pathological, process that leads to extensive tissue destruction. This process has an inflammatory character

(reviewed by Finn 1986) and involves the shedding of all but the basal third of the endometrium. Menstruation is preceded by a fall in progesterone secretion, that directly stimulates myofibroblasts to gather and to secrete latent MMPs, and leukocytes to secrete cytokines to induce an activation of these MMPs (reviewed by Salamonsen & Woolley 1999; reviewed by Kelly *et al.* 2001). Endometrial myofibroblasts are induced by lymphocyte-secreted TNF-α to synthesize IL and prostaglandins (**PG**), that attract neutrophils (reviewed by Kelly *et al.* 2001). The human endometrium is able to undergo this inflammation-like process repeatedly without it resulting in the deposition of scar tissue (reviewed by Salamonsen 2003). This is likely to be a result of intricately regulated collagenolytic activity by MMPs. This physiological process can be used as a model for pathological endometritis, and there is an increased secretion and activation of MMP-2 and MMP-9 in endometrial extracts from dogs with endometritis (Chu Py *et al.* 2002). Therefore, although the role of MMPs during equine endometritis has not previously been investigated, they have been associated with endometritis or comparable processes in other species.

1.3.6.4 The role of uterine MMPs during gestation

The gelatinases and the TIMPs have been studied in human placenta and foetal fluids throughout gestation. It has been found that in the first trimester, MMP-2 and MMP-9 are produced by the trophoblast in close contact with the decidua, associating their activities with the invasive ability of these cells (Polette *et al.* 1994). This is in accordance with a study showing that endometrial invasion by equine chorionic girdle trophoblast cells around Day 34 of gestation requires MMP-2 and MMP-9 (Vagnoni *et al.* 1995). The study also found that whereas MMP-2 activity

persisted, albeit in the stroma and not the trophoblast cells, of anchoring placental villi for the remainder of pregnancy, neither MMP-9 mRNA nor protein could be detected in anchoring villi at term (Polette *et al.* 1994). Furthermore, TIMP-1 and TIMP-2 mRNA and protein was expressed by decidual cells during the first and third trimester, and it was concluded that these inhibitors limit the invasiveness of trophoblast cells into the uterine wall, and that a balance of MMP and TIMP is crucial in the spatial and temporal control of endometrial invasion by the placenta (Polette *et al.* 1994). MMP-2 and TIMP-2 mRNA were both expressed at a comparable level in first and third trimester, whereas a temporal discrepancy took place between the expression of MMP-9 and TIMP-1. Maximal expression of MMP-9 was found during the first trimester, and maximal expression of TIMP-1 was observed during the third trimester (Polette *et al.* 1994). As pregnancy progresses, it is suggested that high levels of TIMP secretion by the placenta bring the invasion of trophoblasts to a halt, maintain tissue integrity and prevent inappropriate tissue breakdown (Huppertz *et al.* 1998; Riley *et al.* 1999b).

1.3.6.5 The role of MMPs during parturition

The establishment of uterine contractions during human parturition coincides with increased MMP-9 over MMP-2 in amniotic fluid, and decreased TIMP-1 levels (Goldman *et al.* 2003). A week before contractions begin, the decidua secretes two types of relaxin, that increase the secretion of latent MMP-9 into the foetal membranes, but do not influence TIMP or MMP-2 (Qin *et al.* 1997a; Qin *et al.* 1997b). In a study on TIMP activity at human parturition, TIMP-2, -3 and -4 immunostaining was intense in the ECM of the amnion, especially immediately

underlying the epithelial BM (Riley *et al.* 1999b). All four TIMPs were found in some, but not all, individual chorionic trophoblast cells, and all but TIMP-1 were localised to the ECM (Riley *et al.* 1999b). This study demonstrated a decrease in TIMP secretion into the amniotic fluid during active labour, suggesting that foetal membrane rupture involved a withdrawal of endogenous inhibition by TIMPs (Riley *et al.* 1999b). It was concluded that the chorion laeve and decidua parietalis are major sites of TIMP secretion throughout pregnancy as levels of TIMPs are low or undetectable in amniotic fluid during first trimester (Riley *et al.* 1999a).

The separation of the placenta from the endometrium requires the action of MMPs, and the activity of latent MMP-9 detected only in the maternal part of the placenta was lower in cows with retained placenta than in controls (Maj & Kankofer 1997). Latent MMP-2 activity was detected in maternal and foetal parts of the placenta and was higher in cows with retained placenta than in controls (Maj & Kankofer 1997). The lack of MMP-9 secretion could play a part in the lack of maturation of placentomes that is observed in placenta retention in cows (Boos *et al.* 2003).

From the above, it can be concluded that MMP-2 and MMP-9 are expressed in the endometrium of the horse and the human among other species, and that their regulation is involved in the remodelling processes that take place during menstruation and pregnancy. During inflammation and the tissue repair that follows, MMP-9 expression is induced by the presence of inflammatory factors, and is involved in the resolution stage of the repair process.

1.4 Events of remodelling in the equine endometrium

The equine oestrous cycle does not involve menstruation, but other uterine events leading to tissue remodelling take place in the reproductively active mare. During her lifespan, a brood mare experiences repeated episodes of breeding, pregnancy, parturition and uterine involution. In this study, it is hypothesised that the deposition of collagen seen in endometrial fibrosis is a result of altered endometrial remodelling, and to understand the aetiology of endometrial fibrosis it is important to investigate physiological and pathological events involving remodelling of the equine endometrium.

1.4.1 Breeding-induced endometritis

Endometritis is a normal physiological event that occurs in the mare following breeding. The equine uterus is well equipped to tackle foreign materials and contaminants, and uterine defences include mechanical, cellular and humoral factors, that are dependent on hormonal status and therefore vary during the oestrous cycle, as will be discussed below.

1.4.1.1 Breeding associated stimuli leading to endometritis

Semen induces an inflammatory response even in the absence of bacteria (Kotilainen *et al.* 1994), but it has been shown that seminal plasma has an immunomodulatory effect in the uterus, resulting in improved pregnancy rates after AI with fresh semen compared to AI using semen extender (Alghamdi *et al.* 2004). It has been shown that seminal plasma reduces sperm-neutrophil binding, exhibited

as a dose-dependent reduction in neutrophil chemotaxis and phagocytosis (Alghamdi *et al.* 2004; Portus *et al*. 2005).

Potentially pathogenic mucosal commensal organisms can also be introduced during breeding, originating either in the stallion or mare genital tracts. The organisms most commonly isolated from the mare and stallion reproductive tracts are beta-haemolytic streptococci, especially *Streptococcus equi* subsp. *zooepidemicus*, or ***S. zooepidemicus*** (Jones *et al.* 1984; Ricketts *et al.* 1993). In a study of a mixed population of mares, bacteria were isolated from 65%, but only 10% of mares had endometritis, as conveyed by concurrent positive cytological and bacteriological findings (Wingfield Digby & Ricketts 1982). In one study stallion semen contained on average approximately 5.7 x 10^5 cfu/ml as shown by microbial culture, although streptococci were isolated in only one ejaculate of 100 examined (Simpson *et al.* 1975). It has been shown that even using aseptical methods, microorganisms can be introduced into the uterus from the posterior part of the genital tract of the mare (McDonnell & Watson 1992), so even using AI it is difficult to prevent the introduction of microorganisms into the uterus. These microorganisms can flourish in the uterus, especially under the influence of progesterone (McDonnell & Watson 1992). A mild, transient endometritis characterised by an increase in protein contents and neutrophil levels in uterine lavage fluid can be provoked solely by physical stimulation of the cervix or uterus through the rectum (Williamson *et al.* 1987).

1.4.1.2 Experimental models of equine endometritis

In 1969, researchers designed experiments to investigate equine endometritis to describe the uterine inflammatory reaction (Hughes & Loy 1969; Peterson *et al.* 1969). By inoculating an actively growing culture of *S. zooepidemicus* into the uterus of young healthy mares, Hughes and Loy (1969) described the uterine inflammatory reaction. Utilizing transrectal palpation, they observed the swelling and oedema of the uterus and cervix. Within 12 hours after inoculation the reaction began to subside and all mares had recovered within 96 hours after inoculation (Hughes & Loy 1969). The experiment demonstrated that young healthy mares are resistant to the establishment of bacterial infection in the uterus during oestrus without a reduction in their fertility. Numerous studies have since been conducted using experimental endometrial models induced by *S. zooepidemicus* inoculations (Ganjam *et al.* 1982; Evans *et al.* 1986; LeBlanc *et al.* 1989; Troedsson *et al.* 1993b; Nikolakopoulos & Watson 1999). Such studies have contributed to the present knowledge about the pathogenesis of equine endometritis.

1.4.1.3 The inflammatory response of the equine uterus

During oestrus the endometrial folds become oedematous, and in some mares this leads to a small amount of intrauterine transudate fluid. This does not appear to influence 1st cycle pregnancy rates (Reilas *et al.* 1997). The folds produce a capillary space in which fluid can be transported toward the cervical opening (Bracher & Allen 1992; Bracher *et al.* 1992). The acute breeding-induced response is characterised by an influx of fluid into the uterine lumen by transudation from the oedematous endometrium (Mitchell *et al.* 1982). As a reaction to uterine insult, an

increased secretion of PGE_2 is seen in uterine fluid, as shown by experimental uterine inoculation with *S. zooepidemicus* (Pycock & Allen 1990). The prostaglandins induce an increased vascular permeability, leading to an influx of fluid into the uterine lumen, containing serum proteins and neutrophils (see **1.4.5.1**). Other inflammatory products secreted by cells in the endometrium include IL -1 and -6, and TNF-α (Fumuso *et al.* 2003). Neutrophils migrate to the uterine lumen as a result of chemotaxis toward inflammatory factors and activated complement, and bind to opsonins and antibodies that have coated the surface of sperm and bacteria. The complements, inflammatory factors and antibodies are produced by resident and infiltrating lymphocytes found in the endometrial stroma. Neutrophil numbers in uterine fluid peak four hours after *S. zooepidemicus* inoculation and a second influx, albeit less dramatic, is seen around 24 hours after inoculation (Williamson *et al.* 1987). A second influx of serum proteins is not seen, as local mucosal production of immunoglobulins is likely to take over to provide opsonising agents (Mitchell *et al.* 1982). An increase in the number of helper T lymphocytes in equine endometrium, as detected by immunohistochemistry, occurred six hours after AI, but numbers had normalised again 48 hours after AI (Tunón *et al.* 1999a). No increase was seen in the numbers of cytotoxic T lymphocytes (Tunón *et al.* 1999a). The primary immunoglobulins (**Ig**) produced in the endometrium are IgA and IgG, that constitute the endometrial mucosal immune response (Mitchell *et al.* 1982). Along with these mechanisms the uterine wall contracts in response to prostaglandins and oxytocin to allow physical clearance of fluid containing inflammatory products and debris (see **1.4.3.2**).

1.4.1.4 Duration of breeding-induced endometritis

When the above mechanisms are fully functional, the inflammatory response provoked by semen is short lived, with neutrophil numbers peaking around eight hours after AI, and with very low numbers of neutrophils found at 48 hours after AI (Katila 1995), although other research has reported neutrophilia in uterine lavage fluid 48 hours after AI (Nikolakopoulos & Watson 1997). When the uterine inflammatory response after natural mating and after fresh semen AI were compared, the amount of intrauterine fluid and neutrophil numbers in intrauterine fluid did not differ (Nikolakopoulos & Watson 1997), showing that the reproductively normal mare is able to clear uterine inflammation even in the presence of possible contaminants in the semen (Simpson *et al.* 1975). The elimination of intrauterine fluid in response to experimentally inoculated *S. zooepidemicus* occurred within 48 hours after uterine inoculation (Nikolakopoulos & Watson 1999).

1.4.1.5 Clinical diagnosis and treatment of endometritis

The clinical diagnosis of endometritis is based on the discovery of intrauterine fluid (>10 mm luminal diameter; (Reilas *et al.* 1997) via transrectal ultrasonography, as well as cytological findings of >5% neutrophils in at least 100 counted cells in endometrial swab samples or uterine fluid (Kotilainen *et al.* 1994; Card 2005). The treatment of endometritis involves a large volume (1-2 litres) intrauterine infusion with sterile physiological saline via the cervix to aid mechanical evacuation of the uterus and flush out potentially harmful uterine contents (see **1.4.5.3**), and the administration of oxytocin to promote myometrial contractions (see

1.4.3.2). In the case of bacterial infection of the uterus, antibiotics can be administered either intrauterine or systemically.

1.4.2. Persistent breeding-induced endometritis

Some mares are not able to rid themselves of the inflammation during the acute stage and tend to retain intrauterine fluid beyond the average time it takes a normal mare to remove it (see **1.4.1.4**), resulting in persistent endometritis. A mare that has the tendency to develop persistent endometritis is called 'susceptible', whereas a mare that does not develop persistent endometritis is termed 'resistant'.

Various methods have been used to determine the susceptibility or resistance of mares to persistent endometritis, such as histopathology, reproductive history and experimental inoculations. Mares with clinical signs of endometritis (see **1.4.1.5**) 96 hours after breeding were considered susceptible to persistent breeding-induced endometritis by Maloufi *et al.* (2002). In another study it was concluded that mares in oestrus do not retain bacterial uterine infections for longer than 18 hours (Watson *et al.* 1987b). From a clinical aspect, it must be considered that the conceptus enters the uterus 120-144 hours (Day 5-6) after ovulation (Betteridge *et al.* 1982), and therefore this is the ultimate time limit for clearance of inflammation in order to ensure the survival of the conceptus. The essential uterine defence mechanisms have been extensively researched in order to determine the key factors influencing resistance and susceptibility to persistent endometritis.

1.4.3 Physical uterine defences

The uterus is physically separated from the environment by the vulva, vagina and cervix that play a role in the uterine defences. The seal of the vulval lips has a role in decreasing the access of contaminants and mares with a slack vulval seal are more prone to vaginitis and resulting endometritis. Vaginitis characterised by eosinophilic infiltrations, that may lead to endometritis, is often seen in mares with pneumovagina due to a loose vulval seal (Slusher *et al.* 1984).

1.4.3.1 Evacuation of intrauterine fluid

Another physical barrier is the cervix that is tightly closed at dioestrus and during gestation and prevents the entry of contaminants. During oestrus the cervix relaxes considerably to allow for the deposition of semen directly into the uterus. This site of deposition is unique to the mare, as in the other domestic species the semen is deposited in the cervix. Due to this anatomical feature the entry of contaminants into the uterus is inevitably easier, and can be expected to occur during breeding. However, the relaxed cervix provides for easy drainage of fluid from the uterus and this is one of the reasons that a mare is better equipped to fight endometritis during oestrus as will be reported below. It is likely that the presence of stagnant fluid in the uterus gives mucosal commensals the optimal conditions for excessive growth and subsequent infection.

Therefore, it is essential that the mechanical evacuation of fluid takes place within the time limits of the acute inflammatory reaction. Susceptible mares accumulated significantly more intrauterine fluid than resistant mares after inoculation with *S.*

zooepidemicus during oestrus, and still had considerable amounts of fluid at the crucial threshold for conceptus survival (see **1.4.2**) on Day 5 after ovulation (LeBlanc *et al.* 1989). It is likely that this fluid would be retained during the ensuing dioestrus due to cervical closure, resulting in a hostile environment for an embryo (Carnevale & Ginther 1992).

1.4.3.2 Role of uterine contractions in fluid evacuation

Uterine contractions facilitate the mechanical evacuation of uterine contents through the cervix or via the lymphatic vessels. This physical clearance is important in the early elimination of bacteria, and is more efficient in resistant than susceptible mares (Evans *et al.* 1987). The endometrium releases large amounts of prostaglandins (PGE_2, PGF) during inflammation (Watson *et al.* 1987c), that in concert with endogenous oxytocin induces myometrial contractions (Capraro *et al.* 1976). Endogenous oxytocin release does not differ between susceptible and resistant mares, but on the administration of exogenous oxytocin the $PGF_2\alpha$ response is less prominent in susceptible mares (Nikolakopoulos *et al.* 2000). Other researchers found that the administration of oxytocin to oestrous mares after intrauterine inoculation of radiocolloid provokes a release of plasma $PGF_2\alpha$ only in mares susceptible to persistent endometritis, and not in resistant mares (Cadario *et al.* 1999). The increased presence of $PGF_2\alpha$ in susceptible mares may be due to the sustained inflammatory uterine environment in these mares (Cadario *et al.* 1999).

1.4.3.3 The influence of uterine stimuli on uterine clearance

In oestrous resistant mares, inoculation of *S. zooepidemicus* during oestrus induces a cervical drainage of material within two hours, resulting in complete clearance within one to two days of inoculation (Evans *et al.* 1986). Susceptible mares are over five times more likely to retain intrauterine fluid 96 hours after uterine challenge with *S. zooepidemicus* than resistant mares (Maloufi *et al.* 2002). When mares receive intrauterine inoculation with microspheres of a size only removable through physical drainage, rapid clearance of material is seen in younger, reproductively healthy mares (Evans *et al.* 1987). A combined inoculum of microspheres and bacteria is cleared more efficiently than charcoal alone, indicating that an antigenic stimulus further enhances the physical clearance mechanism (Evans *et al.* 1987). This increased physical clearance due to an antigenic stimulus is possibly enabled via the effects of $PGF_2\alpha$ (Troedsson 1999). There is no difference in uterine clearance between susceptible and resistant mares when frozen semen or semen extender are introduced into the uterus, and it is assumed that these substances are less inflammatory and less persistent than bacteria (Maloufi *et al.* 2002; Güvenc *et al.* 2004).

1.4.3.4 Effect of reproductive hormones on uterine contractility

It has been shown that the oestrous uterus exhibits short bursts of electromyographic activity of high amplitude as demonstrated by myometrial electrode implants (Jones *et al.* 1991), whereas dioestrous concentrations of plasma progesterone result in sustained low grade electromyographic activity, and an associated retention of antigenic and non-antigenic material (Evans *et al.* 1986;

LeBlanc *et al.* 1989). The evacuation of microspheres was delayed under the influence of progesterone, indicating that this hormone decreases physical uterine clearance (Evans et al 1987). This suppressive effect of progesterone was confirmed by the findings that mares developed bacterial endometritis under the influence of progesterone as a consequence of transcervical manipulation (McDonnell & Watson 1992). After uterine inoculation of oestrous mares with charcoal, resistant mares had cleared the charcoal within 48 hours without accumulating intrauterine fluid, whereas susceptible mares had accumulated fluid and a charcoal coagulum (Kolm *et al.* 2005). When clenbuterol, an inhibitor of smooth muscle activity (β_2 agonist) was administered, smaller amounts of uterine lavage fluid were recovered, possibly representing the reduction in uterine contractions moving fluid accumulations towards the cervix (Kolm *et al.* 2005). In a previous study, electromyographical activity in oestrous mares was not found to be consistently affected by clenbuterol, whereas there was a tendency for suppression during dioestrus (Jones *et al.* 1991). In rabbits, oestrogen promotes uterine contraction, whereas progesterone promotes uterine relaxation via the regulation of α- and β-receptors, possibly explaining this diverging effect of clenbuterol depending on the presence of sex steroids (Roberts *et al.* 1989).

1.4.4 Neutrophil function during endometritis

Neutrophils are an important part of the acute cellular response to uterine insult as they enter the uterine lumen in great numbers and act as the first line of defence regardless of the nature of the stimulus. Endometritis in the mare does not

induce an increase in peripheral white blood cell counts as the leukocyte response takes place locally in the endometrium (Troedsson *et al.* 1993b).

1.4.4.1 Numbers of intrauterine neutrophils during endometritis

Various components of semen induce an influx of neutrophils into the equine uterine lumen (Kotilainen *et al.* 1994). By collecting low volume (50ml) uterine lavages six hours after the introduction of different combinations and concentrations of semen, seminal plasma and semen extenders, the highest numbers of neutrophils were found in reaction to small volumes of highly concentrated semen (Kotilainen *et al.* 1994). In the process of preserving semen, seminal plasma is removed and replaced with smaller volumes of semen extender (see **1.1.4**). Due to the reduced amount of inseminate fluid, spermatozoa can be expected to induce an increased inflammatory response as they adhere to the endometrium. Another consequence of the lack of fluid in inseminate is the reduction of immediate physical drainage that normally happens purely due to the volume of fluid. As already mentioned seminal plasma has an immunomodulatory effect by decreasing the neutrophil-sperm binding (Alghamdi *et al.* 2004). Therefore the lack of seminal plasma in concentrated semen can be expected to lead to increased phagocytic activity of neutrophils as well as increased number of neutrophils. There is no additional inflammatory effect of bacteria introduced during natural breeding when compared with AI with chilled semen (extender with antibiotics added; Nikolakopoulos & Watson 1997). It may therefore be assumed that neutrophil numbers arriving in the resistant equine uterus during endometritis are independent of the type of inflammatory stimulus.

The response of susceptible mares 48 hours after AI using extended semen was reported to be more pronounced than in resistant mares, characterised by larger amounts of intrauterine fluid, larger numbers of neutrophils and more tendency to yield positive bacterial growth from uterine lavages (Nikolakopoulos & Watson 1997). Also, the reduction in neutrophil numbers over the first 36 hours was steeper in resistant mares, indicating a more rapidly subsiding inflammatory reaction than in the susceptible mares (Troedsson *et al.* 1993b). The endometrial expression of IL-6 is increased in susceptible mares, and has an initial promoting effect on inflammation. However, IL-6 later on induces neutrophil apoptosis, possibly explaining the sustained numbers of neutrophils in susceptible mares (Fumuso *et al.* 2003; Kaplanski *et al.* 2003). No differences were found between susceptible and resistant mares in the numbers of neutrophils recovered 96 hours after infusing frozen semen or semen extender alone (Maloufi *et al.* 2002). It is possible that the late sampling during the inflammatory response failed to demonstrate differences that had existed earlier after uterine inoculation, although the differences between resistant and susceptible mares would be expected to become increasingly apparent with time.

1.4.4.2 Function of neutrophils during endometritis

Comparative studies have shown that uterine fluid from susceptible mares contains adequate numbers of neutrophils during induced *S. zooepidemicus* endometritis (Evans *et al.* 1987). As susceptibility did not appear to be determined by a lack in the numbers of uterine neutrophils, their functional capacity was investigated. The function of uterine neutrophils involves the migration to the

uterine lumen as a reaction to immunological factors in uterine fluid (chemotaxis) and the phagocytosis of spermatozoa and bacteria (Asbury *et al.* 1982). Uterine fluid contains immunological factors that are chemotactic to neutrophils, and induce opsonization, the coating of foreign particles by specific proteins to enable phagocytosis (see **1.4.5.2**). Uterine neutrophils from susceptible mares were shown to be fully functional in response to *S. zooepidemicus* inoculation, and even displaying more pronounced chemotaxis and phagocytosis than uterine neutrophils from resistant mares (Troedsson *et al.* 1993b). However, uterine secretions from susceptible mares were a poorer source of opsonin (see **1.4.5.2**), and it was concluded that deficient phagocytosis by neutrophils in susceptible mares was due to dysfunctional opsonization (Troedsson *et al.* 1993b). It was therefore demonstrated that uterine neutrophils from susceptible mares are fully functional in the appropriate environment, confirming the findings by Asbury and Hansen (1987) and directing the focus of study towards the immunological function of uterine fluid.

1.4.5 Immunological function of uterine fluid

An important part of the uterine inflammatory response is the influx of fluid into the uterine lumen. It has been demonstrated that even physical manipulation of the cervix and uterus may initiate a uterine influx of fluid and proteins (Martin *et al.* 1988). Although the accumulation of intrauterine fluid in mares has been correlated with increased pregnancy loss (Troedsson *et al.* 1993b) and is positively correlated with mare age (Kotilainen *et al.* 1994), it contains components essential to the inflammatory response. In reproductively normal mares, there is no difference in

pregnancy rates between mares with or without intrauterine fluid accumulations after AI (Kotilainen *et al.* 1994).

1.4.5.1 Influx of fluid into the uterine lumen

The uterine influx of neutrophils is preceded by an influx of protein (Martin *et al.* 1988), possibly representing chemoattractants inducing neutrophil migration (Blue *et al.* 1984). Serum proteins access the uterine lumen by a combination of passive diffusion, transudation, and active transport and secretion (Mitchell *et al.* 1982). A high albumin concentration in uterine fluid of normal mares indicates that this protein enters the uterine lumen by transudation (Tunón *et al.* 1998). Increased numbers of neutrophils in uterine fluid coincide with the appearance of PGF, PGE_2 and leukotriene (**LT**)B_4 in the uterus (Watson *et al.* 1987a; Watson *et al.* 1987c; Watson *et al.* 1988). Equine endometritis is generally seen as a local inflammation not involving the systemic circulation although, contrary to those beliefs, some chemotactic stimulation appears to occur in the circulation. This was exhibited by the greater degree of chemotaxis shown by blood-derived neutrophils from susceptible mares than resistant mares (Troedsson *et al.* 1993b).

1.4.5.2 Opsonizing ability of uterine fluid

Uterine fluid also has a role in inducing neutrophil phagocytosis of foreign particles and cells by opsonization. Opsonization involves the coating of particles with serum and specific proteins in order to accelerate phagocytosis. Among these proteins are complement components, especially C3 and immunoglobulins, especially IgG (Frank & Fries 1991). There is a large variation in protein

concentrations in uterine fluid within a relatively homogeneous population of reproductively normal mares (Tunón *et al.* 1998). In a study on experimental *S. zooepidemicus* endometritis it was shown that protein concentrations in uterine fluid did not differ significantly between resistant and susceptible mares (Troedsson & Liu 1992). Previous research reported higher amounts of IgA and IgG in uterine secretions from susceptible mares than resistant mares, even in the absence of uterine infection (Asbury *et al.* 1980). A high concentration of IgA in uterine fluid indicates that this immunoglobulin is locally produced and preferentially secreted by the endometrium (Tunón *et al.* 1998). When the uterine specific antibody response was investigated, Ig titres to *S. zooepidemicus* were similar in endometrial culture supernatant from susceptible and resistant mares (Watson & Stokes 1990). However, opsonizing activity of supernatant from susceptible mares was less effective than from resistant mares, possibly reflecting different ratios of the various subclasses of IgG (Watson & Stokes 1990). In a study on complement in uterine fluid from non-infected resistant mares, it was indicated that a portion of uterine opsonizing activity was due to complement, although varying results had been reported previously, possibly due to different assays (Håkansson *et al.* 1993). No difference was found in levels of IgG and C3 in uterine fluid between resistant and susceptible mares during the first 24 hours following *S. zooepidemicus* inoculation (Troedsson *et al.* 1993c), and therefore defective opsonization is likely to result from other opsonizing proteins.

1.4.5.3 Intrauterine fluid accumulations in susceptible mares

During the first 36 hours after an induced infection, susceptible mares accumulate larger amounts of intrauterine fluid than resistant mares (Troedsson & Liu 1992). This accumulation of fluid may have a negative effect on the opsonization properties of the inflammatory products contained in this fluid, for example by complement inactivation or by dilution (Troedsson 1999). This could in part explain the fact that several researchers have found that uterine neutrophils in susceptible mares lose function more quickly than resistant mares (Cheung *et al.* 1985), possibly contributing to the increase seen in bacterial numbers 12 to 96 hours after bacterial inoculation in susceptible, and not resistant mares (Williamson *et al.* 1987). Even if this accumulated fluid contains beneficial inflammatory mediators, it is likely to also harbour destructive products, such as proteolytic enzymes, that could cause tissue damage if not eliminated from the uterine lumen. This tissue damage can possibly lead to endometrial fibrosis, that is often encountered in susceptible mares (Troedsson & Liu 1992).

1.4.5.4 Antibacterial function of uterine fluid

As well as promoting the phagocytosis of bacteria, uterine fluid has non-specific antibacterial activity, due to bactericidal proteins such as lactoferrin and lysozyme as seen in the cow and pig (Roberts *et al.* 1976; Dixon & Gibbons 1979). Lysozyme concentrations in equine uterine fluid are increased during the early post-partum period but are practically absent in non-parturient mares (Reilas & Katila 2002). The human endometrium secretes defensins, small molecules that have antimicrobial actions against bacteria, fungi and viruses (King *et al.* 2003). In a

study on the antibacterial properties of mare uterine fluid, it was demonstrated that the growth of *S. zooepidemicus* was inhibited by dioestrous and not oestrous uterine fluid (Strzemienski *et al.* 1984). In a study on the antibacterial properties of peroxidase in uterine fluid, the activity in the normal equine uterus was insignificant, but was increased following inoculation with *S. zooepidemicus* (Blue *et al.* 1982). Peroxidase is a haem protein, that is important in the bactericidal function of neutrophils, and is spilled into the extracellular space during extensive phagocytosis (reviewed by Malle *et al.* 2007), and therefore it is possible that the bactericidal activity of uterine fluid during oestrus is attributable to neutrophils, rather than a direct effect of fluid proteins.

1.4.6 Effect of reproductive hormones on uterine fluid and neutrophils

Blood-derived neutrophils from oestrogen treated mares are more phagocytic than neutrophils from progesterone treated mares 48 hours post-inoculation (Ganjam *et al.* 1982). This is also true for uterine neutrophils with increased phagocytic activity under the influence of oestrogen, and decreased activity in the presence of progesterone (Watson *et al.* 1987b; Watson & Stokes 1988). Blue and co-workers found no difference in protein content of uterine fluid depending on the stage of the oestrous cycle, but *in vitro* tests revealed that neutrophils incubated in dioestrous fluid were less phagocytic than neutrophils incubated in oestrous fluid or in saline controls (Blue *et al.* 1982). As described in **1.1.2.2**, important changes occur in genital anatomy during oestrus, including cervical relaxation, uterine folds becoming more prominent (Bracher & Allen 1992) and increased myometrial contractions (Troedsson *et al.* 1993d), all acting to aid in uterine clearance (Evans *et al.* 1986).

1.4.7 Summary of uterine defence mechanisms

To summarise, the normal uterine response to semen and mucosal bacteria during breeding involves the influx of uterine fluid, large numbers of neutrophils, immunoglobulins and other proteins. In resistant mares, the inflammation is resolved within 48-96 hours. In mares failing to clear the inflammation within this time, many aspects of uterine defences have been investigated. The physical clearance of intrauterine fluid seems to be a crucial point in separating resistant and susceptible mares, as accumulated uterine fluid in susceptible mares seems to be deficient in opsonising capacity and lead to premature loss of neutrophil function, with ongoing inflammation as a result.

The inflammatory response that takes place in the equine uterus as a consequence of breeding is normally short-lived. In some mares this response becomes chronic, persisting until and after the conceptus arrives in the uterus, leading to pregnancy loss. Even if the inflammation is mild, the chronic injury occurring to the endometrium is likely to lead to a change in the regulatory mechanisms of tissue remodelling and repair, including the regulation of the MMPs and TIMPs

1.5 Summary and hypothesis

To summarise, equine endometrial fibrosis is a condition that significantly reduces the fertility of mares with advancing age. The condition is characterised by periglandular organization of myofibroblast-like cells, that then proceed to deposit excess amounts of periglandular collagen, and in some cases a general stromal fibrosis can result. These descriptions in many respects are reminiscent of

histological descriptions of pathological fibrosis in liver, lung and other organs. The fibrosis of these organs has been researched and the pathogenesis described as the disruption of the wound repair and ECM remodelling following chronic injury. MMPs are important in the normal physiological remodelling that ensues following tissue injury, and have been found to be involved in the excess deposition of collagen in the liver and lung. In the breeding mare, the endometrium comes into contact with contaminants at the time of breeding, provoking a transient physiological inflammatory response. However, in some mares this inflammation tends to become chronic. Other physiological remodelling events occur in the equine endometrium during pregnancy, that involves the communication with the conceptus, the invasion of endometrial cups and placental development and growth.

Therefore, it is hypothesised that in the normal mare, the activity of MMP-2 and MMP-9 (gelatinases) and the TIMPs is involved in endometrial remodelling events during physiological events such as breeding-induced endometritis and pregnancy, and that the alteration in the activity of these enzymes leads to fibrosis of the equine endometrium.

1.5.1 Aims of the study

To investigate this hypothesis, the main aims of the study were:

- To study the histological distribution of leukocytes residing in the equine endometrium during the normal oestrous cycle and during endometritis, and correlate these with the detection of MMP-2, MMP-9 and TIMPs.

- To determine the activities of MMP-2 and MMP-9 (gelatinases), and TIMPs in the equine endometrium during the normal oestrous cycle and during endometritis.

- To investigate if Icelandic mares bred using an extensive management system exhibit signs of CDE, and if the Kenney category system is useful in this population. Additionally it is hoped to investigate if the amount of endometrial collagen deposits in the equine endometrium can be correlated with the activities of MMP-2 and MMP-9 (gelatinases), and TIMPs and any of the aetiological risk factors previously suggested.

- To investigate the activities of MMP-2 and MMP-9 (gelatinases), and TIMPs in equine foetal fluids during gestation and during parturition and to determine if there is a correlation between these activities and the outcome of gestation.

2 Chapter 2:

Materials and methods

2 Materials and methods

2.1 Collection of samples from non-pregnant mares

2.1.1 Preparation of mares

Before samples were collected the mares underwent a physical examination, as well as a reproductive examination including transrectal palpation and ultrasonography to establish the stage of their oestrous cycle. Before proceeding with the examination, a swab was collected from the clitoral fossa as described in **2.1.3.1**, after which the tail was wrapped in a long rectal examination glove and held to one side. Wearing a long plastic sleeve over one arm, the clinician emptied the rectum of faecal balls before proceeding to palpate and scan the genital tract transrectally. From mares used in the experimental protocol reported in chapters **3** and **4** vaginal (see **2.1.3.2**) and endometrial swabs (see **2.1.3.3**), as well as endometrial biopsies (see **2.1.6**) were collected at the beginning of the protocol to ascertain their suitability for the experiment (see **3.1.1** and **4.1.1**).

Transrectal palpation and ultrasonography were carried out daily until the stage of cycle had been established, and then every other or third day unless required more frequently in correlation with induction of endometritis, sampling or treatment. The first day of oestrus was determined by the presence of one or more ovarian follicles with a minimum diameter of 35mm coinciding with oedema of the uterine wall (see **1.1.2.2**) identified by transrectal ultrasonography. The follicle was monitored until the day of ovulation that was considered to be day 0 of the oestrus cycle. During dioestrus, clinical findings demonstrated using transrectal ultrasonography were

defined as the presence of one or more CL coinciding with an absence of uterine oedema.

2.1.2 Clinical examination

Clinical findings as observed, palpated and identified via transrectal ultrasonography were recorded before carrying out any manipulations of the reproductive tract. Discharge from the vulva was recorded either as being present or absent. The diameter of follicles and presence of corpora lutea were recorded for both ovaries as observed on transrectal ultrasonography. Uterine oedema as seen on transrectal ultrasonography was scored on a scale 0-3 (0: no oedema; 1: oedema; 2: prominent oedema; 3: very prominent oedema). The maximum diameter of intrauterine fluid was measured using callipers on the scanner display and scored on a scale of 0-3 (0: no uterine fluid detected; 1: diameter $\leq$ 10 mm; 2: diameter of 11 to 39 mm; 3: diameter $\geq$ 40 mm). The degree of echogenicity (the reflectivity of ultrasound by the uterine contents) was scored on a scale of 1-4, adapted from (McKinnon *et al.* 1993) with 1=anaechoic and 4=hyperechoic. Increased echogenicity indicates increased numbers of cells, especially neutrophils, in uterine fluid and is therefore a sign of endometritis. During oestrus, it was accepted that intrauterine fluid score 1 did not necessarily indicate endometritis (Reilas *et al.* 1997), however any amount of fluid during dioestrus indicated endometritis. For a confirmed diagnosis of endometritis, the intrauterine fluid accumulations were required to coincide with positive cytological findings in endometrial swab samples with or without a positive microbiological culture from endometrial swabs (see **2.1.3.4**).

2.1.3 Collection of swabs

Following examination and prior to sample collection through the vagina the mare's perineal area was washed by applying povidone-iodine surgical solution and rinsing with clean water, repeated three times, and dried with clean paper towels. For all transvaginal procedures, one hand and arm were covered with a long plastic sleeve and the hand was covered with a sterile surgical glove lubricated with sterile K-Y Jelly (K-Y Brand; Johnson & Johnson Ltd).

2.1.3.1 Collection of clitoral swabs

A swab sample was collected from the clitoral fossa in all mares at the beginning of the experimental protocol (chapters **3** and **4**) in order to determine if the contagious equine metritis (**CEM**) organism was present. Contagious equine metritis is a venereal disease in horses that is notifiable to the World Organization for Animal Health (Anon 2006). This was carried out by rolling a fine swab in the clitoral fossa surrounding the clitoral body before the perineal area had been washed. The swab was transported in Amies charcoal medium (Transwab ENT Charcoal; Medical Wire & Equipment) as instructed by the World Organization for Animal Health (Anon 2006) and cultured within 1 hour.

2.1.3.2 Collection of vaginal swabs

Vaginal swabs were collected from each mare at the beginning of the experimental protocol (chapters **3** and **4**) to confirm the absence of vaginitis. A swab was introduced through the vulva and advanced to the cranial part of the vagina by the external cervical opening. About 5 cm from the cervical opening, the swab was

rolled gently on the vaginal mucosa and retracted in the palm of the hand to protect it from contamination. The swab was rolled on a microscopy slide and prepared for cytological analysis (**2.1.3.4**).

2.1.3.3 Collection of endometrial swabs

Using a sterile gloved hand as described in **2.1.3**, a sterile double-guarded swab (Equivet; Kruuse) was guided manually through the vulva, vagina and cervix to reach the uterine body. After the swab was positioned in the uterine body, it was rolled on the endometrial surface for 30 sec. The swab was retracted into the protecting tube of the double-guarded swab and removed from the mare. The swab was then rolled gently on a sterile glass microscopic slide to spread material collected for cytology (see **2.1.3.4**), after which it was retracted within the inner protective tube, sealed with sterile plastic caps at both ends and processed for microbiological culture within 1 hour.

2.1.3.4 Analysis of smears

For cytological preparation, smears were air dried for 10-20 min and processed using Diff-Quick stain (Medion Diagnostics AG). One hundred cells were counted in each smear at high power (400X) magnification and were classified as either neutrophils or other cells. The presence of over 5% neutrophils in a minimum of 100 cells was interpreted as a sign of endometritis (Card 2005), that was confirmed by the coinciding clinical observation of intrauterine fluid collections as described in **2.1.2**.

Identification of microbial pathogens was carried out using conventional microbiology procedures. Endometrial cytology in combination with uterine aerobic culture is a reliable method of diagnosing bacterial endometritis (Reiswig *et al.* 1993). The criterion for diagnosing a pathological genital infection in mares or stallion was a growth of over 10 bacterial colonies in monoculture from swab samples (Hinrichs *et al.* 1988), coinciding with a presence of over 5% neutrophils.

2.1.4 Collection of penile swabs from the stallion

Before washing the penis, swabs were collected from the preputial folds, penile shaft, glans and urethral fossa and submitted to microbiological culture within 1 hour. A mixed flora of skin and mucosal dwelling organisms was considered normal physiological occurrence (Jones *et al.* 1984) and infection was only suspected if a monoculture of a potential pathogen was detected. A swab for CEM was also collected from the urethral fossa and preserved in Amies charcoal medium for microbiological culture (see **2.1.3.1**).

2.1.5 Collection of uterine lavages

After preparing the perineal area a catheter was inserted through the cervix and into the uterus and isotonic infusion fluid was introduced into the uterus. Uterine lavages analysed for chapter **4** were collected using a sterile silicone balloon tip catheter (65 cm; Equivet, Kruuse) to introduce 60ml of pre-warmed sterile isotonic infusion fluid (Isolec; Vetivex, Arnolds Veterinary Products) into the uterine lumen using a syringe. Uterine lavages analysed for chapter **5** were collected using a sterile **pvc** (polyvinyl chloride) uterine catheter (190 cm; Equivet, Kruuse) connected to a

500ml bag of pre-warmed sterile isotonic saline (Icepharma) and introducing the fluid into the uterine lumen by gravity and compression of the bag. Once the fluid was in the uterine lumen the uterus was massaged via the rectum for approx 30 sec and then as much as possible of the fluid was removed via the catheter, either by gentle suction of a syringe (chapter **4**) or by gravity (chapter **5**). A 15ml representative sample of the uterine lavage was collected in a centrifuge tube and spun at 2000 x g for 15 min. The supernatant was collected in a clean tube and stored at -20°C.

2.1.6 Collection of endometrial biopsies

After uterine lavages had been collected, endometrial biopsies were collected as described by Ricketts (1975a) via the cervix using alligator jaw biopsy forceps with a 4 x 15 mm basket (62 cm; Equivet, Kruuse). After introducing the forceps through the cervix the left hand was introduced into the rectum whilst holding the forceps handle with the right hand, keeping the basket closed. After locating either body-horn junction of the uterus the jaws were turned to the side and opened while pushing down with one finger of the left hand so that a fold of endometrial tissue would get caught between the jaws. The fold was pinched off and the forceps were extracted whilst keeping the basket closed.

2.1.6.1 Frozen biopsies for in situ *zymography*

Frozen biopsies from each control biopsy and each induction model in each mare were prepared for *in situ* zymography for chapter **4** (see **2.5.1**). A piece approximately 5 mm long was cut from the biopsy and placed on a small amount of

optimal cutting temperature (**OCT**) compound (Tissue-Tek; Sakura Finetek) on a labelled cork disc and covered over with OCT compound. The cork disc was then placed on a slurry mixture of isopentane and dry ice until the OCT compound froze over. The disc was then turned so the sample and OCT compound rested against the slurry mixture for a few minutes. The discs were then immediately transferred for storage into a -70°C freezer, taking care that the samples did not thaw whilst transferring them.

2.1.6.2 Formalin fixed biopsies for histology and immunohistochemistry

Biopsies were also fixed in formol saline for histological staining (H&E, **2.6.2**; picrosirius, **2.6.3**) and immunohistochemistry for MMP-2 and MMP-9 (see **2.7.1**). While the samples in **2.1.6.1** were freezing over, the remaining 10 mm long piece of the biopsy was immersed in 10% formol saline. Fixation was allowed for at least 24 hours, after which the tissue was washed in 70% ethanol, followed by 100% ethanol, before being embedded in paraffin wax.

2.2 Induction of endometritis

For chapters **3** and **4**, experimental models were designed to represent the hormone dependent differences in uterine reaction to streptococci, by inducing endometritis during oestrus and during dioestrus using streptococci inoculations of the uterus (see **2.2.2**). To provide a model of the reaction occurring after natural breeding, mares were mated to a stallion when they were in sexually receptive oestrus (see **2.2.3**).

2.2.1 Experimental design

The experimental design chosen was a crossover case-control study, with each animal serving as its own internal control. Uterine lavages (see **2.1.5**) and endometrial biopsies (see **2.1.6**) were collected from each mare. Each mare provided control samples at day one of oestrus (see **2.1.1**) and day nine of dioestrus, after which experimental endometritis was induced. The study was performed under the approval of the University of Edinburgh Animal Ethics Committee and project licence obtained under the Home Office Animals (Scientific Procedures) Act 1986. The animals were kept outside on a drylot and fed haylage.

2.2.2 Induction of streptococcal endometritis

Streptococcal endometritis was induced by introducing an inoculum of *S. zooepidemicus* into the uterus via the cervix. To prepare the inoculum, a pure culture of *S. zooepidemicus* previously isolated from equine endometritis and stored at -20°C was cultured on 5% sheep blood agar at 37°C overnight under aerobic conditions. Calibration was carried out at a wavelength of 600 nm in a spectrophotometer (Cecil CE 2021; Cecil Instruments Ltd) to determine the relationship between light absorption and number of colony forming units (**cfu**) in a suspension of the culture.

The day before a mare was ready for inoculation an overnight culture was carried out as described above and an inoculum was prepared immediately before depositing into a mare. Colonies were scraped off the heavy culture plates using a sterile loop and suspended in sterile phosphate-buffered saline (**PBS**). The suspension was measured in the spectrophotometer and calibrated so that it contained a magnitude of

10^6 cfu and 1ml of this suspension was put into 29ml of sterile isotonic infusion fluid (Isolec; Vetivex, Arnolds Veterinary Products).

The reproductive tract was monitored by transrectal ultrasonography and once the mare was at the desired stage of the oestrous cycle (first day of oestrus or ninth day of dioestrus; see **2.1.1**) an inoculum of *S. zooepidemicus* was prepared. Prior to introducing the inoculum an endometrial swab was taken to make certain that mares were free of infection (see **2.1.3.3** and **2.1.3.4**). After preparing the perineal area (see **2.1.3**) the 30ml inoculum was introduced into the uterus through the cervix via a sterile insemination pipette (600 mm; Bovivet, Kruuse), using air to empty the pipette into the uterus. The few drops that remained in the pipette after inoculation were taken for microbiological culture to ascertain the purity of the culture.

Five hours after introducing the inoculum a clinical inspection was made to observe signs of endometritis: vulvar discharge, intrauterine fluid and increased oedema via transrectal ultrasonography (see **2.1.2**). After clinical observation at five hours post inoculation, uterine lavage samples were collected and prepared (see **2.1.5**). Twenty hours post inoculation clinical signs were observed again after which uterine lavages and endometrial biopsies (see **2.1.6**) were collected. After sample collection the mares were monitored for clinical signs of endometritis (see **2.1.2**) and endometrial swabs were collected for cytology and microbiology to ascertain that the infection was resolved (see **2.1.3.3** and **2.1.3.4**) before proceeding with the protocol.

2.2.3 Induction of breeding-induced endometritis

At oestrus, after confirming that the mares were free from endometrial infection four mares were mated once each to the same 14 year old crossbred stallion. Prior to the first mating session it was confirmed that the stallion was free from any pathogenic genital infections as described in **2.1.4**. Before each mating the mare was teased (presented to the stallion) to ascertain that she was in sexually receptive oestrus. This was verified by observing the following signs: squatting, urinating and eversion of vulvar labia (Squires 1993) After that the stallion's penis was washed in warm clean water starting distally and working up towards the prepuce, then dried before bringing the stallion to the mare to reduce the number of mucosal organisms introduced during mating (Jones *et al.* 1984).

Uterine lavage samples (see **2.1.5**) were collected from the mares five hours after mating. Uterine lavages and endometrial biopsies (see **2.1.6**) were then collected 20 hours post mating. The mares were monitored via transrectal ultrasonography in the following days for clinical signs of endometritis (see **2.1.2**) and to determine the day of ovulation. On day 6-7 post ovulation they were administered $PGF_{2\alpha}$ to terminate a potential pregnancy by luteolysis. At the end of the endometritis induction protocol a named veterinary surgeon certified that the mares were in good health after which they were discharged and returned to stock.

2.2.4 Treatment of endometritis

During streptococcal endometritis in dioestrus, mares received uterine treatment the day after the second set of samples had been collected to ensure

recovery before proceeding with the protocol. Uterine treatment consisted of large volume (1-2 litre) uterine lavage with isotonic infusion fluid (Isolec; Vetivex, Arnolds Veterinary Products) followed by an i.v. administration of oxytocin (10-20 IU Oxytocin; Intervet).

2.3 Collection of foetal fluids from pregnant mares

Three separate groups of mares were used to provide samples of foetal fluids. One group of pony mares had been chronically catheterised as a part of a previous study carried out by another research group and archive samples of amniotic fluid (kindly provided by Prof Abigail L Fowden, Cambridge University) from this study were used to represent amniotic fluids throughout pregnancy. A second group of pony mares provided allantoic and amniotic fluid samples collected during foaling for this study. These samples were kindly collected by Franziska Palm and Prof Christine Aurich at the University of Vienna. The third group were thoroughbred mares that provided allantoic and amniotic fluids during foaling. The samples were kindly collected for this study by Mr Sidney Ricketts, Rossdale & Partners, Newmarket.

2.3.1 Collection of amniotic fluid from chronically catheterised mares

Amniotic catheters were established in pregnant pony mares, enabling repeated sample collections of amniotic fluid throughout gestation. For gestational stages of mares, see **6.1.1**. Food, but not water, was withdrawn 18 hours before surgery, and the cyclooxygenase inhibitor, meclofenamic acid (2mg/kg; Arquel V Granules; Pfizer Ltd), was given orally the night before surgery and twice daily for 2 days thereafter to reduce endogenous prostaglandin production associated with

fasting and surgery (Silver *et al.* 1979). The mares were premedicated and then anaesthetised with a bolus dose of ketamine (2mg/kg bolus) followed by a continuous i.v. infusion of propofol (0.13-0.20mg/kg per minute; Rapinovet; Schering-Plough Animal Health) as described previously (Taylor et al. 1992). After induction of anaesthesia, the mare was placed in right lateral recumbency, and the uterus was exposed through a midline abdominal incision. A series of small incisions were made sequentially through the uterus, placenta and amnion. Before placing a polyvinyl catheter (outer diameter, 1.52 mm; inner diameter, 0.68 mm; Critchley Electrical Products Ltd) in the amniotic cavity, polyvinyl catheters were inserted into the dorsal aorta and caudal vena cava of the foetus via the tarsal artery and vein (Ousey et al. 2003). The amnion was closed by tying its edges around the catheters using linen thread (5.0 Metric Size 2; Barbour Threads Ltd). A catheter was then inserted into the common umbilical vein. The placenta and uterine incisions were closed using resorbable sutures (Dexon 3.5 Metric Dexon-II BiColour; Braun-Dexon). A uterine vein draining the area close to the incision site was then catheterised. The peritoneum and abdominal layers were closed sequentially using resorbable sutures. Through a second incision in the flank, a catheter was inserted into the maternal dorsal aorta and exteriorised through the same stab wound in the flank as the other catheters. Finally, the skin incisions were closed with nylon (Prolene, Ethicon, 3.5 Metric; Johnson & Johnson Ltd). An antibiotic was given i.v. to the foetus at the end of surgery (ampicillin, 25mg/kg; Penbritin; Beecham Animal Health) and to the mare (ampicillin, 1g) on the day of surgery and for 3 days thereafter. Patency of the foetal catheters was maintained by continuous infusion of heparin-saline (heparin, 200 IU/ml in 0.9% [wt/vol] NaCl; infusion rate,

2.5ml/day) using small, portable pumps (Graseby Medical Ltd) housed in a bag secured to the flank of the mare. Normal feeding patterns were generally resumed within 24-36 hours after surgery. The animals were sampled between 09:00 and 10:00 on 1 to 10 day intervals, with a 5ml sample of amniotic fluid being collected each time and prepared as described in **2.3.3**.

2.3.2 Collection of foetal fluids from foaling mares

Pregnant mares at term were observed by grooms or by researchers and when foaling commenced, foetal fluids were collected in clean containers and prepared as described in **2.3.3**. Allantoic fluid was collected into sterile 15ml centrifuge vials at the spontaneous rupture of the chorioallantoic membrane, taking care not to contaminate the fluid sample. Once the allantoic membrane had ruptured, sterile amniotic samples were withdrawn into a syringe by needle puncture of the amnion within some minutes of its appearance through the vulva, and then aliquoted into sterile 15ml vials.

2.3.3 Preparation of foetal fluid samples

The foetal fluid samples were centrifuged at 2000 x g for 15 min and the supernatant was collected into another sterile 15ml vial. All samples were stored at −20 °C until they were analysed by gelatin zymography (see **2.4.3**) and reverse zymography (see **2.4.4**).

2.4 Electrophoresis based techniques

In order to determine MMP-2 and MMP-9 activity in uterine lavages and foetal fluids, gelatin zymography was used. TIMP activity in uterine lavages and foetal fluids was determined with reverse zymography. Both of these methods utilize the principle of electrophoresis to separate molecules of different molecular weight.

The electrophoretic mobility of a charged molecule depends on the net charge of the molecule, size and shape of the molecule, and the strength (voltage) of the electrical field used. To be able to separate molecules strictly by their size the effect of their net charge on their movement must be eliminated. The **SDS-PAGE** (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) technique uses SDS as an ionic detergent that dissociates proteins into their individual polypeptide sub-units and gives a uniform net charge along the denatured polypeptide. As a result the molecules migrate strictly according to polypeptide size, making it possible to determine their molecular mass by comparing their mobility to polypeptides of known molecular mass run under the same conditions.

2.4.1 Preparing SDS-PAGE gels

A discontinuous system was used for this study, involving a resolving gel topped with a stacking gel in which samples are loaded (for recipes, see **Appendix II**). The Bio-Rad minigel system (Bio-Rad) was used to prepare a resolving gel, topped with sec-Butanol to create a level top to the gel. This was washed off and the gel was left overnight to equilibrate, topped with stacking gel buffer diluted 1:4 with water. After washing off the buffer a stacking gel was poured on top with spacers

forming wells. The stacking gel was then left for 90 min to set. The gel apparatus was then filled with running tank buffer and the samples were loaded into the wells. Broad range molecular markers of roughly 30-210 kiloDaltons (**kDa**; Precision Plus Protein Standards; Bio-Rad) were always loaded on 7.5% acrylamide gels used for gelatin zymography (see **2.4.3**). Low range molecular markers of roughly 22-110 kDa (Precision Plus Protein Standards; Bio-Rad) were loaded on 12% acrylamide gels used for reverse zymography (see **2.4.4**). Gels were run at a constant voltage of 100V for the appropriate length of time, until the samples were seen to be 1-2 mm off the lower edge of the gel.

2.4.2 Sample preparation for electrophoresis

Uterine flushing samples stored at -20°C were thawed and 1ml aliquoted into 2ml Sarstedt tubes, ready to be concentrated by freeze drying and reconstituting in distilled water. Each Sarstedt tube containing 1ml of uterine flushing sample was covered with parafilm with 3-5 small holes pierced in it, and left at -70°C overnight. The tubes were then placed in a suitable rack in the freeze drier and vacuum pump apparatus and freeze-dried at a pressure of 8-10 mbar until dry (overnight for 1ml). The pressure was then released and samples were ready to be reconstituted in a known volume of distilled water or else stored capped at -20°C. Allantoic and amniotic fluids did not require to be concentrated up and were used directly after preparing and storing as described in **2.3.3**.

2.4.3 Detection of MMP-2 and MMP-9 using gelatin zymography

Gelatin zymography detects the activity of latent and active forms of the gelatinases, MMP-2 and MMP-9 using a method described previously (Heussen & Dowdle 1980), adapted and routinely used by this laboratory (Riley *et al.* 1999a). The resolving gel contains gelatin, a substrate for the gelatinases. Proteins are visualised as a site of enzymatic activity, identified as a clear band in a dark-stained gel, as the gelatin will have been digested at this site. The latent pro-forms of the gelatinases are detected by this method due to SDS in the gel exposing the active site via a conformational change, as well as dissociating active forms from their inhibitors.

2.4.3.1 Sample application and zymography protocol

After preparing gels (see **2.4.1**) the samples mixed 1:1 with sample application buffer were loaded into the wells (for recipes, see **Appendix II**). Used as a control was a sample of human amniotic fluid collected at term spontaneous delivery, characterised extensively by this laboratory previously (Riley *et al.* 1999a) and containing latent pro-forms and active forms of MMP-2 and MMP-9. Gels underwent electrophoresis in running tank buffer at 100 V for approximately 90 min. Gels were washed twice (first for 15 min, then 45 min) with Triton-X wash buffer, then twice (2 min each) with tris-buffered saline (**TBS**) wash buffer, then incubated in zymography digestion buffer for 18 hours at 37°C. In order to verify the metalloproteinase origin of the gelatinolytic activity, representative gels were also incubated with 2.5mM and 10mM 1,10-phenanthroline, and 5mM and 20mM EDTA (see **1.3.1**) added to the digestion buffer. Afterwards the gels were washed twice (2

min each) with TBS buffer, immersed in Coomassie Blue staining solution for 3 hours at room temperature. Gels were then immersed in de-staining solution for 30 min, followed by 60 min to reveal separate bands where gelatin had been hydrolysed by gelatinase activity.

2.4.4 Detection of TIMPs using reverse zymography

Reverse zymography detects the activity of TIMPs as previously described (Edwards *et al.* 1996). Samples are separated by SDS-PAGE as described in **2.4**, using gels containing gelatin and an MMP preparation collected from BHK-21 cells (kindly provided by Prof Dylan R Edwards, University of East Anglia). Upon incubation, the MMP preparation in the resolving gel degrades the gelatin substrate, and the TIMP proteins are visualised as lack of gelatinolytic activity in site of relevant molecular weight.

2.4.4.1 Sample application and reverse zymography protocol

Gels were prepared (recipes, see **Appendix II**) and loaded with samples mixed 1:1 with sample application buffer, then run for approximately 140 min at a voltage of 100V. As a control, a sample of human amniotic fluid collected during caesarean section at term was used, that had been characterised extensively by this laboratory previously (Riley *et al.* 1999a) and expressing TIMP-1, -2 and -3. The gels were washed in Triton-X wash buffer and incubated in reverse zymography digestion buffer for 17 hours at 37°C, then washed twice with TBS wash buffer. As for gelatin zymography the gels were stained for 3 hours using Coomassie Blue staining solution then de-stained with de-staining solution for 4-5 hours, or until dark

bands of TIMPs were distinct. The presence of TIMPs was detected by their discrete inhibition of MMP activity, seen as a dark band on a lighter background. TIMPs were identified and characterised by comparison with molecular weight markers, and the already characterised human amniotic fluid control.

2.4.5 Analysis of zymography and reverse zymography gels

Zymography and reverse zymography gels were scanned (GS-800 scanner; Bio-Rad) and semi-quantified by densitometry using Quantity One software (Version 4.6.3; Bio-Rad).

2.5 Detection of gelatinase activity in tissue sections using *in situ* zymography

Gelatin substrate zymography measures all forms (latent MMPs and active MMPs) of the gelatinases and therefore does not reliably represent true physiological activity that is influenced by the presence of MMP inhibitors and activators, or its location in the tissue. The principle of *in situ* zymography involves a specific enzymatic substrate detectable by microscopy that is put into contact with cryostat sections of unfixed tissue. When this substrate undergoes lysis as a consequence of enzymatic activity it enables the investigator to localize sites of enzymatic activity in the tissue (Ratnikov *et al.* 2000).

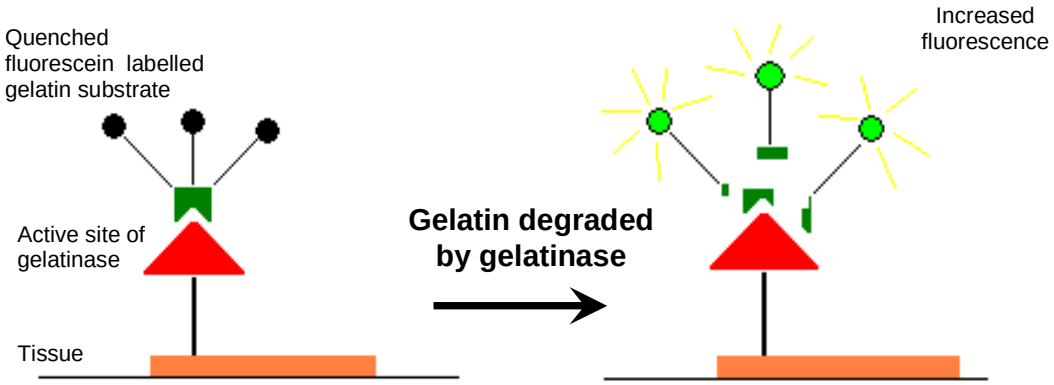


Figure 2.1 The principle of in situ zymography. Quenched fluorescein labelled gelatin substrate is bound by the active site of a gelatinase (MMP-2 or MMP-9). When the gelatinase degrades the gelatin substrate, smaller subunits of gelatin are formed with increased levels of fluorescence (Yi *et al.* 2001).

The substrate used in this protocol was porcine gelatin conjugated to a fluorescent tag (DQ gelatin from pig skin, fluorescein conjugate; Molecular Probes). The gelatin is so heavily labelled with fluorescein that the fluorescence is quenched (**Figure 2.1**). This gelatin substrate is placed on top of the frozen section of the unfixed tissue. When the tissue section is incubated at 37°C, the substrate will be digested in a time- and dose-dependent manner by the appropriate activated enzymes in their native location. When the substrate has been digested, highly fluorescent peptides are released, resulting in an increase in fluorescence in distinct sites where active gelatinases are found in the tissue (Yi *et al.* 2001).

2.5.1 *In situ* zymography protocol

Sections of frozen endometrial biopsies were cut to 7µm on a -18°C cryostat then mounted on SuperFrost Plus glass slides (Menzel-Gläser) and stored in airtight containers at 5°C until used to prevent them drying out. The protocol was carried out

in Shandon Sequenza staining racks (Thermo Scientific) and sections were wet using PBS when mounting. The fluorescein conjugated gelatin substrate was added at a concentration of 10µg/ml to a mixture of 2% gelatin and 2% sucrose in PBS (for recipes, see **Appendix II**) after incubation in a 55°C waterbath for 10 min. 100µl of this mixture was added to each tissue section and incubated at 37°C overnight in a humidified chamber. The following day nuclear counterstain was carried out following 6 washes with PBS at 37°C. DAPI nuclear stain (Sigma) was used at 1:1000 and incubated for 10 min at room temperature. After washing off the nuclear stain with PBS the slides were mounted using permafluor (Beckman Coulter Ltd). Sections were then analysed by confocal microscopy.

To verify the metalloproteinase nature of the detected gelatinase activity, inhibitory action of EDTA and 1,10-phenanthroline was investigated by two different incubation methods. Sections were either preincubated at 37°C with different concentrations of inhibitors for varying lengths of time (1, 3, 6, 24 and 48 hours) prior to overnight incubation with the substrate or incubated at 37°C with different concentrations of inhibitors added in the substrate. The concentrations used for EDTA were 5mM and 20mM and the concentrations of 1,10-phenanthroline were 2.5mM and 10mM.

To distinguish between the gelatinolytic activities of MMP-2 and MMP-9, specific inhibitors were also applied to cryostat sections. Oleic acid (MMP-2 inhibitor I; Calbiochem) was used to inhibit MMP-2 and Anthranilic acid (MMP-9 inhibitor I; Calbiochem) was used as a specific inhibitor of MMP-9. Oleic acid was

preincubated at 37°C for 24 hours at 1µM, 10µM and 100µM prior to overnight incubation with the substrate. Anthranilic acid was preincubated at 37°C for 24 hours at 5nM, 50nM and 5µM prior to overnight incubation with the substrate.

2.5.2 Immunolocalization of neutrophil myeloperoxidase in endometrium

In an attempt to identify cells expressing gelatinase activity as neutrophils, indirect immunohistochemistry (see **2.7**) was used to localize neutrophil myeloperoxidase (**MPO**) before sections were subjected to *in situ* zymography. The primary MPO antibody was polyclonal rabbit anti-human MPO (Dako) and it was detected using goat anti-rabbit fluorescent secondary antibody (Alexa 546; Invitrogen Ltd). Negative controls were carried out by substituting the primary antibody with a matching concentration of rabbit IgG.

Sections were blocked in 3% H_2O_2 for 30 min at 20°C and in normal goat serum for 45 min. The primary MPO antibody was diluted at 1:2000 in 10% normal goat serum and incubated on cryostat sections for 3 hours at 20°C. The secondary fluorescent antibody, diluted at 1:200 in PBS were added to the sections and incubated for 45 min at 20°C. Sections were then subjected to *in situ* zymography (see **2.5.1**).

2.6 Histological preparation of endometrial biopsies

2.6.1 Preparation of endometrial sections

Paraffin wax blocks of endometrial tissue fixed in formol saline were placed face down on an ice block for 30 min prior to cutting to ease cutting of thin sections.

A microtome was used to cut sections of 5μm. When a ribbon of sections was achieved it was transferred to a water bath at 37°C to flatten out the sections and separate them. Separate sections were mounted on SuperFrost Plus glass slides (Menzel-Gläser) and incubated at 37°C overnight to dry.

2.6.2 Haematoxylin and Eosin stain

Sections were dewaxed in histoclear for 5 min, rehydrated in graded ethanol (100% EtOH, 95% EtOH, 70% EtOH, 50% EtOH 2 min each) before being rinsed in tap water prior to haematoxylin staining for 5 min. The slides were rinsed briefly in acid-alcohol and rinsed immediately in H_2O. The slides were dipped in Scott's solution and washed in H_2O before being dehydrated in graded ethanol (50% EtOH, 70% EtOH, 95% EtOH, 100% EtOH 2 min each; histoclear 5 min; xylene 5 min) then mounted with coverslips using Pertex.

2.6.3 Quantitative analysis of collagen fibres using picrosirius red-staining

Picrosirius polarization can be utilised to visualize collagen fibres in tissues, as this dye reacts with collagen. When observed through Polaroid filters, picrosirius stained sections exhibit enhanced birefringency that is considered specific to collagen types I, II and III (Junqueira 1979).

2.6.3.1 Picrosirius staining of endometrial sections

Five micron formol saline fixed sections were dewaxed, rehydrated and stained in haematoxylin. Slides were washed and placed for 1 hour in a 0.1% solution of Sirius Red (Direct Red 80; Sigma) dissolved in aqueous saturated picric

acid. Slides were washed in two changes of acetic acid water, dehydrated, cleared and mounted in Pertex mounting medium.

2.6.3.2 Quantitative analysis of collagen fibres

To assess the amount of collagen in endometrial biopsies, the sections were evaluated using polarization microscopy. A microscope (Leica Microsystems GmbH) was set up with Polaroid filters above the objective lens (static) and below the condenser (with the ability to rotate) and a colour video camera (3CCD; JVC (UK) Ltd) attachment linked to a Macintosh computer. The computer software package used was Scion Image (Scion Corporation). Ten non-overlapping images of glandular areas were taken per section at 160X objective magnification, avoiding areas of artefactual damage, starting at one end of the sections and recording every third field encountered when moving through the section until 10 images had been recorded. The colour images (**Figure 2.2 a** and **b**) were converted to greyscale (**Figure 2.2 c** and **d**) using Adobe Photoshop 3.0 (Adobe Systems Inc) software before being transferred to Quantity One software (Bio-Rad) for optical density analysis. In order to analyse the amount of collagen in the stroma, glandular areas were excluded, and density readings were obtained for the area analysed, resulting in values for the optical density of each mm^2 of stroma.

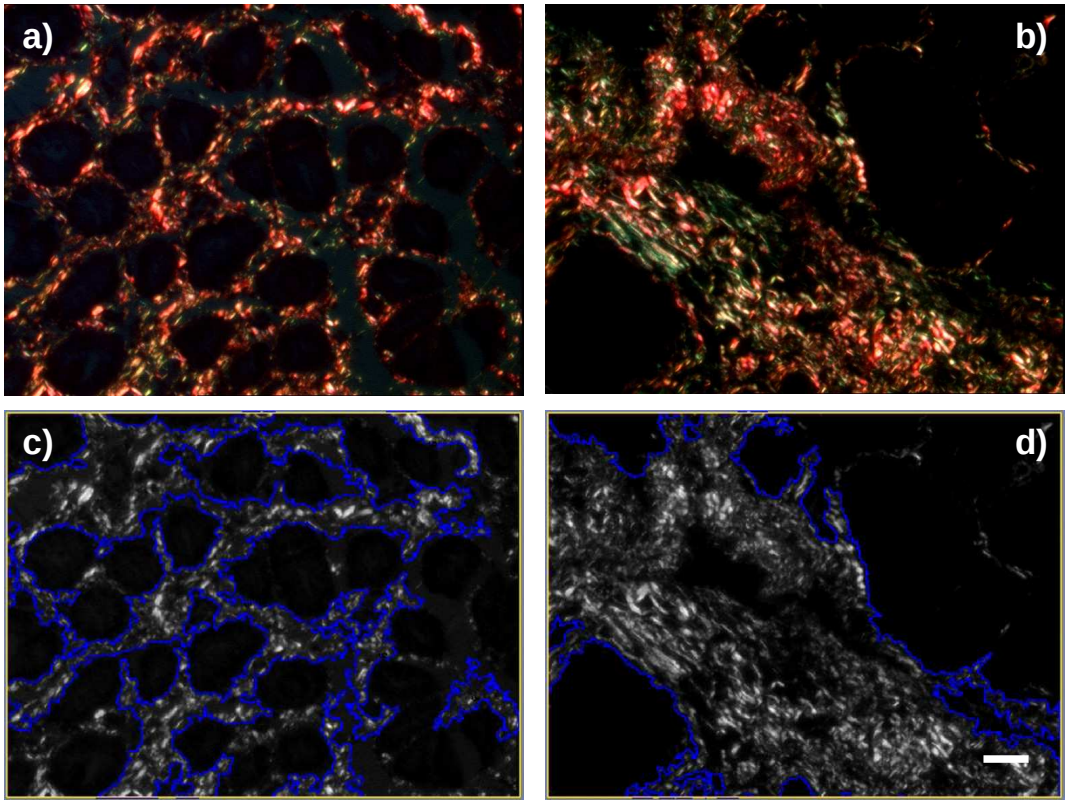


Figure 2.2 Representative photomicrographs demonstrating birefringent collagen fibres in equine endometrium. The photomicrographs are taken using polarised light to demonstrate collagen fibres in equine endometrial biopsy sections stained with picrosirius. Photographs a and b are colour photographs of two different biopsy sections. Photographs c and d have been transformed into greyscale and the stromal areas have been selected by drawing around them in blue, to selectively analyse the optical density emitted by the collagen fibres. Scale bar: 50µm.

2.7 MMP immunohistochemistry and western blotting

The methods of immunohistochemistry and western blotting both utilize the antibody-antigen reaction to detect target molecules in tissue or fluid samples. Indirect immunohistochemistry involves an unlabelled primary antibody (first layer), that reacts with the target antigen, and a labelled secondary antibody (second layer), that reacts with the primary antibody. The secondary antibody must be against the animal species in which the primary antibody was raised. In a common procedure, a biotinylated secondary antibody is coupled with streptavidin-horseradish peroxidase (**HRP**). To visualize the antigen-antibody complex the HPR reacts with detection

reagents. In this study two reagents were used; enhanced chemiluminescence (**ECL**) detection reagent to emit light (**Figure 2.3**), making it possible to develop photography films of the expression pattern of the protein, and 3,3'-Diaminobenzidine (**DAB**) to produce a brown staining (**Figure 2.4**).

2.7.1 Immunohistochemistry for detection of MMP-2 and MMP-9

To detect MMP-2 and MMP-9 in equine endometrial and placental tissues, monoclonal mouse anti-MMP-9 (Insight Biotechnology Ltd) and monoclonal mouse anti-MMP-2 (Calbiochem) were used. Placental tissues used were from the archives of Mr Sidney W Ricketts, Newmarket, and had been collected after spontaneous abortions in thoroughbred mares and fixed in Bouin's solution. The same source also provided identical endometrial sections that had been fixed in Bouin's and formol saline, so that a comparison of the suitability for immunohistochemistry could be made between the two fixation methods. After carrying out the protocol on equine tissues, it appeared that fixation had masked MMP-2 and MMP-9 antigens in the tissues, leading to very weak and inconsistent staining. In order to unmask the antigens the sections were subjected to two different methods of antigen retrieval; heating in a microwave and digestion by trypsin (MacIntyre 2001). Trypsin digestion resulted in total absence of staining whereas microwaving appeared to enhance specific staining of endometrial tissue, and was therefore included in the protocol as described in **2.7.1.1**.

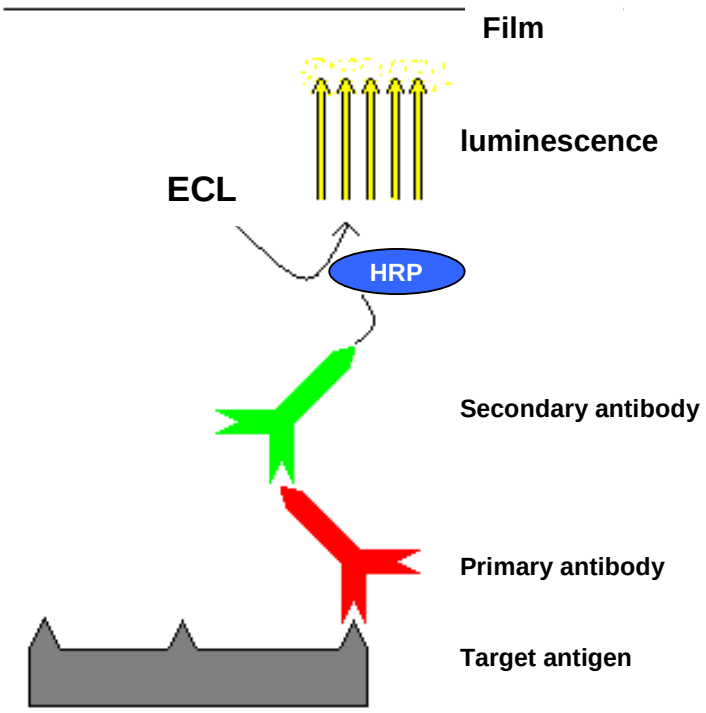


Figure 1.3 The principle of ECL (enhanced chemiluminescence). The detection of antibody-antigen complexes is based on the production of chemiluminescence by applying a chemical reagent to the HRP (horseradish peroxidase) label on a secondary antibody that recognises the primary antibody to the target antigen.

2.7.1.1 MMP-2 and MMP-9 immunohistochemistry protocol

Tissue sections were dewaxed in histoclear for 5 min, rehydrated in graded ethanol (100% EtOH; 95% EtOH; 70% EtOH; 50% EtOH; 2 min each). Antigen retrieval was carried out by microwaving sections at 700W in a tri sodium citrate buffer (for recipes, see **Appendix II**) for 2 min, then resting them in the buffer solution for 15 min. Endogenous peroxidase activity was inhibited by incubation in H_2O_2 (3% (v/v) solution in H_2O) for 60 min. Sections were washed in TBS (2 x 5 min) and blocked in horse serum for 30 min followed by the primary antibody (diluted 1:250 in normal horse serum) overnight at 4°C. The slides were washed in TBS (2 x 5 min) before adding biotinylated secondary horse anti-mouse antibody

(Vector Laboratories) for 30 min. After 2 x 5 min washes in TBS, the streptavidin-horseradish peroxidase complex (ABC; Vectastain, Vector Laboratories) was added for 30 min. The slides were washed in TBS (2 x 5 min) and the DAB substrate (Vector Laboratories) was added to detect positive staining by producing brown stain. Colour development was monitored microscopically and stopped by washing stains in H_2O. Cells were lightly counterstained in haematoxylin (10 sec), washed in H_2O, dipped briefly in acid-alcohol and rinsed immediately in H_2O. The slides were dipped in Scott's solution and washed in H_2O before being dehydrated in graded ethanol (50% EtOH 2 min, 70% EtOH 2 min, 95% EtOH 2 min, 100% EtOH 2 min, histoclear 5 min, xylene 5 min) then mounted with coverslips using Pertex. Negative controls were performed in parallel in which normal horse serum was added to sections instead of the primary antibody.

2.7.1.2 Results of MMP-2 and MMP-9 immunohistochemistry

Bouin's fixed tissue proved inappropriate for MMP-9 immunohistochemistry using mouse anti-human monoclonal primary antibody due to moderately strong non-specific staining of the endometrial stroma. When antigen retrieval was applied by microwaving sections, staining was still non-specific, but slightly weaker than without antigen retrieval. Formol saline fixed endometrial sections exhibited weak non-specific staining against MMP-9 when antigen retrieval was not applied, but after microwaving what appeared to be specific staining of glandular epithelium was detected. However, staining throughout the tissue was uneven and inconsistent. Sections of placenta exhibited some, but inconsistent MMP-9 positive staining of the trophoblast cells when antigen retrieval was applied.

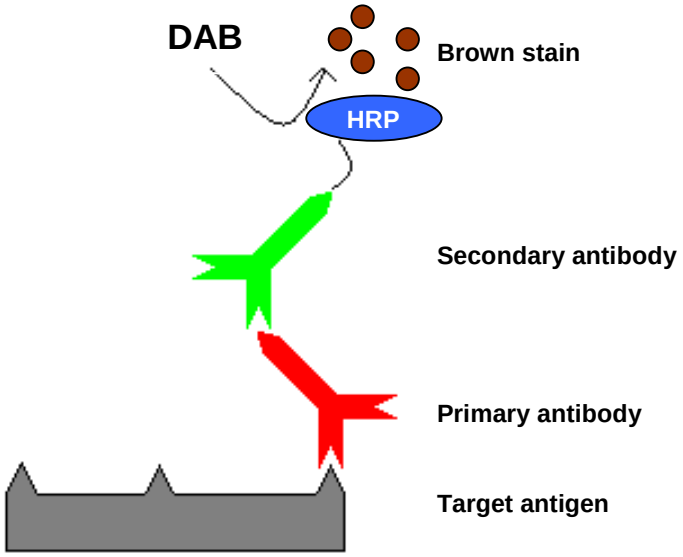


Figure 2.4 The principle of DAB (3,3'-Diaminobenzidine). The detection of antibody-antigen complexes is based on the production of brown stain by applying a chemical reagent to the HRP (horseradish peroxidase) label on a secondary antibody that recognises the primary antibody to the target antigen.

2.7.2 Western blotting for MMP-2 and MMP-9

Due to the inconsistent response of equine endometrium and placenta to MMP-2 and MMP-9 antibodies (see **2.7.1.3**), western blotting was carried out to positively identify MMP-2 and MMP-9 in equine uterine lavages and amniotic fluid by antigen recognition. The protocol is well characterised and routinely used in this laboratory (Riley *et al.* 2000). Western blotting separates proteins by electrophoresis for then to identify them by immunodetection. In this study the DAB and ECL methods were used for immunodetection (see **2.7**). A control sample was human amniotic fluid collected at spontaneous vaginal delivery at term previously characterised by this laboratory to be immunopositive for MMP-2 and MMP-9 as determined by Western blotting (Riley *et al.* 2000). In this laboratory, human

samples are routinely reduced by boiling them in sample application buffer, that optimizes the anionic charge of the protein and reduces protein disulfide bonds to ease protein recognition by the antibody (Wan *et al.* 1995). Some antibodies only recognize the native protein, so reducing the protein reduces antigen recognition. When equine uterine lavage fluid samples were reduced, no appropriate bands were demonstrated by MMP-9 staining, and therefore the protocol was adapted so that equine samples were not reduced even though the human controls were reduced.

2.7.2.1 Protocol

The primary antibodies used in this protocol were the same MMP-2 and MMP-9 antibodies as used in **2.7.1**. Additionally, a polyclonal rabbit anti-MMP-9 (Sigma) was applied, in an attempt to elucidate variable results due to the secondary anti-mouse antibody. A resolving gel and stacking gel were prepared as described in **2.4.1** (for recipes, see **Appendix II**). Samples of uterine lavages and amniotic fluid that had exhibited gelatinolytic activity on gelatin zymography at the molecular weights corresponding to MMP-2 and MMP-9 were loaded onto the gel for then to be run at 100V for 100 min. Once electrophoresis was completed, the gel was immersed in transfer buffer for 30 min, ready to be transferred onto a nitro-cellulose membrane (pore size 0.45µm; Bio-Rad). A gel sandwich was assembled, taking care to keep all components wet with transfer buffer at all times. The gel sandwich was kept on an ice pack during the transfer at a voltage of 100V for 60 min.

After transfer, the membrane was immersed in a blocking solution containing 5% bovine serum albumin (**BSA**; Sigma) in tween tris-buffered saline (**TTBS**), for 60

min and then stored at 5°C overnight. Next, primary MMP-2 or MMP-9 antibody (both at 1:1000 concentration in 10ml blocking solution plus 0.025% Na Azide; Merck) were added to the membrane and incubated for 2 hours at 20°C. Negative controls were carried out by incubating a corresponding membrane in blocking solution without the added primary antibody to determine if the secondary antibody exhibited non-specific binding to the membrane. The membrane was cut into two identical halves, so that each half could be subjected to a different detection method.

DAB detection

To one half of the membrane a secondary biotinylated antibody was added (sheep anti-mouse diluted 1:200 for mouse anti-MMP-2 and mouse anti-MMP-9; Vector Laboratories, goat anti-rabbit diluted 1:2000 for rabbit anti-MMP-9; Amersham) and incubated at 20°C for 45 min. DAB (Vector Laboratories) detection was carried out on the membrane, and when desired intensity of staining was reached, the membrane was immersed in water to stop the staining reaction.

ECL detection

To the other half of the membrane an HRP linked secondary antibody (sheep anti-mouse diluted 1:2000 for mouse anti-MMP-2 and mouse anti-MMP-9; Amersham, donkey anti-rabbit for rabbit anti-MMP-9; Amersham) and incubated at 20°C for 1 hour. After washing the membrane in TTBS, ECL reagent (Amersham) was added and incubated for 1 min. The membrane was then taken to the dark room and used to illuminate photographic film in order to produce a representative photography of MMP-2 and MMP-9 positive bands. Exposure times varying from

20 sec to 30 min were applied to the film, using developer and fixer reagents and film from Kodak (Sigma).

2.7.2.2 Results of western blotting for MMP-2 and MMP-9

When equine samples were reduced by boiling in sample application buffer, no positive staining for MMP-9 appeared in the 100 kDa region however there was a positive MMP-2 reaction in the 60 kDa region. MMP-9 positive bands were seen around 60-70 kDa. This indicated that MMP-9 might be binding non-specifically to proteins on the membrane. It was decided not to reduce the equine samples that resulted in detection of MMP-9 in the 92 kDa region when the DAB technique was used, but not when using the ECL method. Additionally, varying degrees of a positive reaction were discovered in negative controls. This implied that the secondary HRP antibody might exhibit non-specific binding to other antigens in the samples. Therefore, MMP-9 identification using rabbit polyclonal primary antibody was attempted. Again there appeared to be a cross reaction between MMP-2 and MMP-9, as the MMP-9 antibody was positive around 60 kDa. To investigate this, human amniotic fluid samples extensively characterised by this laboratory samples, with one containing only MMP-2 and the other containing only MMP-9, were run side by side. Non reduced samples of equine uterine lavages were also added to this gel. The human samples revealed MMP-9 positivity only in the 100 kDa region, confirming the specificity of the antibody to human MMP-9. Only vague immunoreactivity to mouse and rabbit anti-MMP-9 antibodies was demonstrated in some of the equine samples, in the 60 kDa and 120-180 kDa regions, none of them corresponding to the molecular weight of human MMP-9.

2.7.3 Conclusion

It was concluded that Bouin's fixative was inappropriate for MMP-2 and MMP-9 immunohistochemistry and therefore formol saline fixed tissues were preferred. Formol saline fixed equine endometrium required antigen retrieval by microwaving to facilitate the detection of MMP-9, and appeared to exhibit specific staining, although the staining tended to be unevenly distributed throughout the tissue. The specificity of trophoblast cells in equine placenta to MMP-9 seemed to be accurate although areas in the tissue sections varied in specificity and staining intensity, from negative to positive. The mouse monoclonal antibody to MMP-2 appeared to be specific to equine tissue samples.

Due to varying results from immunohistochemistry, western blots were performed to assess the validity of anti-human MMP-2 and MMP-9 antibodies. Equine uterine lavages shown to contain gelatinolytic activity at the molecular weights corresponding to MMP-2 and MMP-9 were analysed and compared with human positive controls. Monoclonal and polyclonal MMP-9 antibodies did not bind specifically to equine MMP-9, and some cross-reactivity seemed to occur with MMP-2 protein. Although MMP-2 appeared specific, the immunostaining exhibited by equine tissues and fluid samples must be considered unreliable.

It was therefore concluded that the anti-human MMP-2 and MMP-9 antibodies available in this laboratory were not appropriate for the detection of the equine isotopes. This was shown by varying results on equine endometrial and placental sections as well as the absence of immunoreactivity with proteins of the right

molecular weight in equine uterine lavage fluid, in spite of positive results on human amniotic fluids run simultaneously. These results therefore do not feature in the main results section of this thesis.

3 Chapter 3: Endometrial leukocyte infiltrations in equine endometritis

3 Endometrial leukocyte infiltrations in equine endometritis

Endometrial remodelling is limited in the cyclic non-pregnant mare but will occur following endometritis that is commonly experienced by breeding mares (Watson 2000). As discussed in **1.4.1.1**, breeding-induced endometritis can occur as a reaction to bacteria and semen, or purely as a reaction to semen (Kotilainen *et al.* 1994). Under the influence of oestrogen, the uterus is well equipped to clear a uterine inflammation, but during dioestrus, the influence of progesterone results in a reduction in the efficacy of uterine defence mechanisms (Evans *et al.* 1986; McDonnell & Watson 1992).

The cellular mechanisms and histopathological features involved in equine endometritis have been described for diagnostic purposes (Kenney 1978). There are no previous studies investigating the influence of type of inflammatory stimulus on the density of endometrial cellular infiltration during endometritis; nor has the potential influence of the stage of the oestrous cycle been examined. In order to investigate the remodelling events involved in equine endometritis, a study was carried out to quantify the inflammatory cells involved in the repair mechanisms occurring in the endometrium during streptococcal and breeding-induced endometritis.

3.1 Materials and methods

3.1.1 Animals and sample collection

Five mares of various breeds, ranging in age from 4 to 12 years and weighing 430 to 700 kg were studied through four consecutive spontaneous oestrous cycles. The mares were reproductively healthy as confirmed by clinical examination (see **2.1.2**), the absence of signs of inflammation or infection in clitoral, vaginal and endometrial swabs (see **2.1.3**) and a histopathological assessment category I-IIA (Kenney and Doig 1986) of H&E stained sections of endometrial biopsies (see **2.6.2**). Endometritis was induced in all mares in oestrus and dioestrus using uterine inoculation with *S. zooepidemicus* (see **2.2.2**) and during oestrus by mating four of the mares to a stallion (see **2.2.3**). One 14 year old crossbred stallion was used for natural mating, and was confirmed to be free from pathological genital infections by microbiological culture of penile swabs (see **2.1.4**).

3.1.2 Clinical assessment

Clinical findings were recorded as described in **2.1.2** before carrying out any manipulations of the reproductive tract at each stage of the protocol. Five hours after inducing endometritis, clinical findings were again recorded before collecting 60ml uterine lavages (see **2.1.5**; chapter **4**), and again 20 hours after inducing endometritis, before collecting uterine lavages (see **2.1.5**; chapter **4**) and formalin-fixed endometrial biopsies (see **2.1.6**). After the collection of samples, mares were examined daily to monitor the progression of endometritis by transrectal palpation and ultrasonography (see **2.1.2**).

3.1.3 Histopathology assessment

Biopsy sections were fixed in formalin (see **2.1.6.1**), stained with H&E (see **2.6.2**) and assessed microscopically for general signs of inflammation, such as oedema and infiltration of inflammatory cells. Numbers of inflammatory cells (lymphocytes, plasma cells, neutrophils and eosinophils) in luminal epithelium, stratum compactum and stratum spongiosum were then determined as follows. Ten high power fields (400X) in each biopsy were counted using a computerised image analysis program (Image-Pro Plus version 4.0; Media Cybernetics). For each field, net areas of luminal epithelium, stratum compactum and stratum spongiosum were calculated (in mm^2). In the case of stratum spongiosum the net area was determined by subtracting the glandular area from the overall tissue area. The number of each cell type in each of ten fields and the area of the corresponding field were recorded and used to calculate a final density for each cell type.

3.1.4 Statistical analysis

Due to each biopsy yielding analyses from ten fields (repeated sampling), generalised linear mixed effect models were used for statistical analysis (Pinheiro & Bates 2000). The animal from which the biopsy was taken was entered into the models as a random effect. The stage of each endometritis induction model (control, during streptococcal endometritis and during breeding-induced endometritis) and the stage of the oestrous cycle (oestrus or dioestrus) were entered as the fixed effect. To take into account that cell numbers (i.e. integers) were being analysed, and some fields yielded no cells, Poisson errors were used. Prior to carrying out the analysis, the relationship between the area analysed and cell numbers was investigated using

Poisson generalised linear models for the three layers of the endometrium (luminal epithelium, stratum compactum and stratum spongiosum) and the four cell types (lymphocytes, neutrophils, eosinophils, plasma cells). This demonstrated that for 11 out of the 12 relationships there was a statistically significant positive relationship. Therefore the area associated with each cell number was entered into the model as a covariate fixed effect. The analyses were carried out to compare cell numbers (lymphocytes, neutrophils, eosinophils and plasma cells) between the different endometritis models (control, during streptococcal endometritis and during breeding-induced endometritis), and the stage of the oestrous cycle (oestrus and dioestrus). For some cell types, no cells were found in any fields in control samples, making statistical analysis impossible. The statistical software package used was R 2.4.1 (Free Software Foundation Inc., Boston, MA, USA) and statistical significance was taken when $p<0.05$.

To investigate if basal cell numbers varied depending on the stage of the cycle, the numbers of cells of each type were compared between oestrus and dioestrus control samples. To investigate if an increase in cell numbers occurred during induced endometritis, numbers of each cell type in each of the endometritis induction models were compared with the corresponding controls (oestrus control compared with streptococcal endometritis in oestrus and breeding-induced endometritis, and dioestrus control compared with streptococcal endometritis in dioestrus). To determine if there were differences in cell numbers provoked by each of the different induction models (streptococcal endometritis in oestrus and in dioestrus, and

breeding-induced endometritis in oestrus), the numbers of each cell type were compared between the three induction models.

3.2 Results

3.2.1 Clinical findings

Clinical findings recorded prior to endometritis induction, and five hours and 20 hours later are shown in **Table 3.1**. All mares were free from clinical signs of endometritis at the collection of control samples but in all cases challenges with *S. zooepidemicus* or natural mating successfully provoked acute endometritis.

Table 3.1 Clinical findings in mares with induced endometritis. Findings are expressed as number of mares and % of total. PI: post inoculation

	5 h PI oestrus	20 h PI oestrus	5 h after breeding	20 h after breeding	5 h PI dioestrus	20 h PI dioestrus
Vulvar discharge	2 (40%)	0	1 (25%)	0	1 (20%)	1 (20%)
Uterine oedema	5 (100%)	5 (100%)	4 (100%)	4 (100%)	5 (100%)	5 (100%)
Uterine fluid	3 (60%)	5 (100%)	1 (25%)	3 (75%)	2 (40%)	3 (60%)
Echoic fluid	2 (40%)	0	1 (25%)	0	2 (40%)	3 (60%)

Before inducing streptococcal endometritis in oestrus, transrectal ultrasonography revealed that all mares had uterine oedema and none had intrauterine fluid. Three mares had developed intrauterine fluid five hours post inoculation that in two mares was echoic (see **2.1.2**) with scores 2 and 3, respectively, and coupled with a purulent discharge from the vulva in the latter mare. All mares had anechoic intrauterine fluid 20 hours post inoculation and no mares had vulvar discharge. Four mares were spontaneously free from endometritis within an average of 53 hours post inoculation (45-72 hours). One mare required uterine lavage and oxytocin treatment to aid uterine clearance (see **2.2.4**) due to an inflammatory reaction caused by an

endometrial swab breaking off and being recovered 70 hours later, resulting in her convalescence 72 hours after retrieving the swab.

Prior to uterine streptococcal inoculation in dioestrus no uterine oedema or intrauterine fluid were detected but oedema was present five and 20 hours post inoculation. Slightly echoic intrauterine fluid was seen in two mares five hours post inoculation and in three mares 20 hours post inoculation. Vulvar discharge was seen in only one mare, both five and 20 hours post inoculation. After the last set of samples had been collected all mares with streptococcal endometritis in dioestrus received a large volume uterine lavage and oxytocin treatment to aid uterine clearance (see **2.2.4**). All mares were free from signs of inflammation after an average of 77 hours post inoculation (36-118 hours).

Before being bred to the stallion, all mares had uterine oedema without intrauterine fluid. Three mares responded positively to teasing with the stallion (see **2.2.3**) but one young and inexperienced mare had vague responses although ultrasound scan of her uterus and ovaries showed her clearly to be in oestrus. This mare required more time to receive the stallion, but eventually stood still while being mounted by the stallion. Once they had been bred all still had uterine oedema five and 20 hours after breeding. One mare had intrauterine fluid five hours after breeding, and three mares had developed fluid 20 hours after breeding, anechoic in all cases. Only one mare had vulvar discharge five hours after breeding that looked like reflux of semen and had resolved 20 hours after breeding.

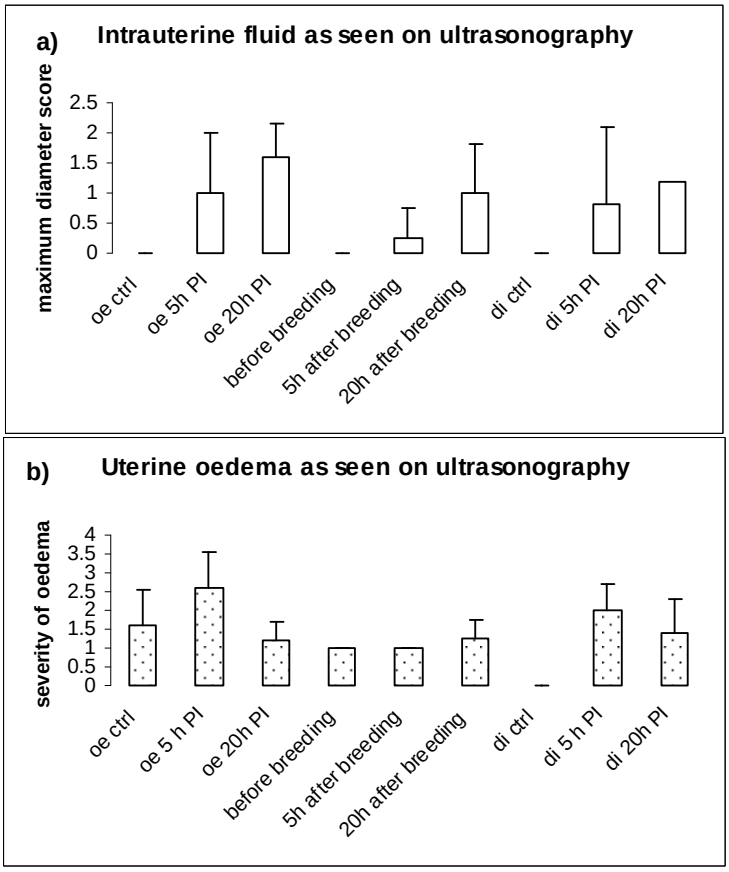


Figure 3.1 Intrauterine fluid and uterine oedema as seen on transrectal ultrasonography. a) Maximum diameter score (no uterine fluid detected, score 0; diameter ≤ 10 mm, score 1; diameter of 11 to 39 mm, score 2; diameter ≥ 40 mm, score 3) for intrauterine fluid before, and 5 and 20 hours after induction of endometritis. b) Uterine oedema score (0: no oedema; 1: oedema; 2: prominent oedema; 3: very prominent oedema) before, and 5 and 20 hours after induction of endometritis. oe: oestrus, PI: post-inoculation with *S. zooepidemicus* inoculation, breeding: natural mating to a stallion.

When the scores for intrauterine fluid were analysed (**Figure 3.1 a**) it was observed that in all induction models, intrauterine fluid was seen between five and 20 hours after induction, with the largest amount occurring during *S. zooepidemicus* endometritis at oestrus. The least amount of fluid was observed five hours after breeding but fluid had increased 20 hours after breeding. Considerable individual variation was observed between mares at corresponding stages of endometritis.

Oedema of the uterine wall as detected by transrectal ultrasonography was increased five hours post inoculation with *S. zooepidemicus* during oestrus and dioestrus but had decreased again 20 hours post inoculation. Five hours after breeding no difference was observed in uterine oedema, whereas it had increased slightly 20 hours after breeding (**Figure 3.1 b**). Individual variation in uterine oedema between mares was less than that observed for intrauterine fluid.

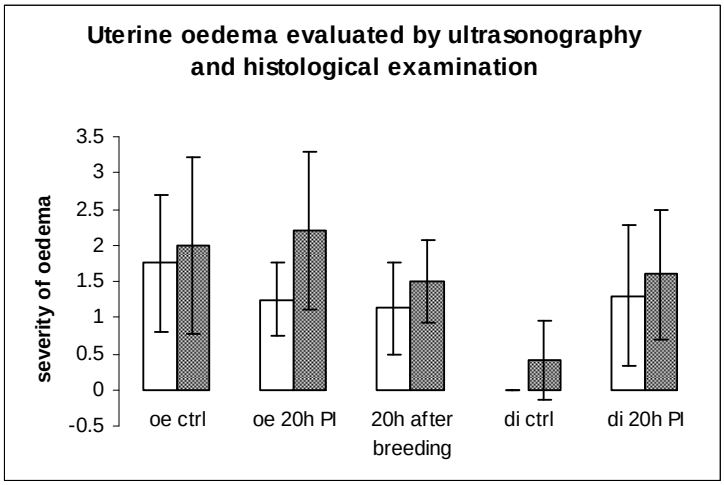


Figure 3.2 Comparative histogram of uterine oedema scores. 0: no oedema; 1: oedema; 2: prominent oedema; 3: very prominent oedema as evaluated by transrectal ultrasonographical examination of the uterus (open bars) and histological examination of endometrial biopsies (stippled bars). Both methods show considerable variation between mares and the general pattern is similar, although the histological examination tends to yield higher scores.

3.2.2 Histopathological assessment

Slight lymphocytic infiltration was seen in most control biopsies collected in oestrus and dioestrus, as well as the occasional slightly distended gland and periglandular fibrosis. During oestrus, the luminal epithelium was medium to tall columnar with apical tufts and basal vacuoles; in dioestrus it was low to medium columnar. Oedema was present in the stratum spongiosum during oestrus and

endometritis. In two dioestrous mares, some unevenly spread oedema was seen in the stratum spongiosum. The severity of histological oedema varied between mares and the development of oedema was similar to what was observed by ultrasonography, albeit scores were consistently higher when scored by histology (**Figure 3.2**). The oedema seen in streptococcal endometritis induced during dioestrus was mostly localised to the upper stratum spongiosum. As a general trend, oedema was most pronounced in streptococcal endometritis induced during oestrus and least apparent following natural mating during oestrus. The cellular infiltrates in oestrus and dioestrus controls (**Figure 3.3 a and b**) were generally minimal to mild, and the pattern was mostly diffuse, although there were very occasional lymphocytic foci in the stratum compactum or stratum spongiosum. No neutrophils were present in oestrus control samples but they were noted, if rarely, in the stratum compactum of dioestrus control samples from three mares. Eosinophils were present in all three layers of oestrus control samples from two mares. They were absent from all but one dioestrus control sample, in which they percolated through the stratum spongiosum.

During streptococcal endometritis in oestrus (**Figure 3.4 a**) the lymphocytic infiltrate was also diffuse but denser, mostly occupying the stratum compactum and upper stratum spongiosum, thought still with occasional more intense foci. Neutrophils were present in both the stratum compactum and stratum spongiosum of all mares, and in all but one mare there were neutrophils transmigrating across the luminal epithelium. During streptococcal induced endometritis in dioestrus (**Figure 3.4 b**) neutrophils and lymphocytes infiltrated both stratum compactum and stratum spongiosum in four mares while in the fifth only the stratum compactum was

infiltrated. Neutrophilic transmigration across the luminal epithelium was seen in all but one mare.

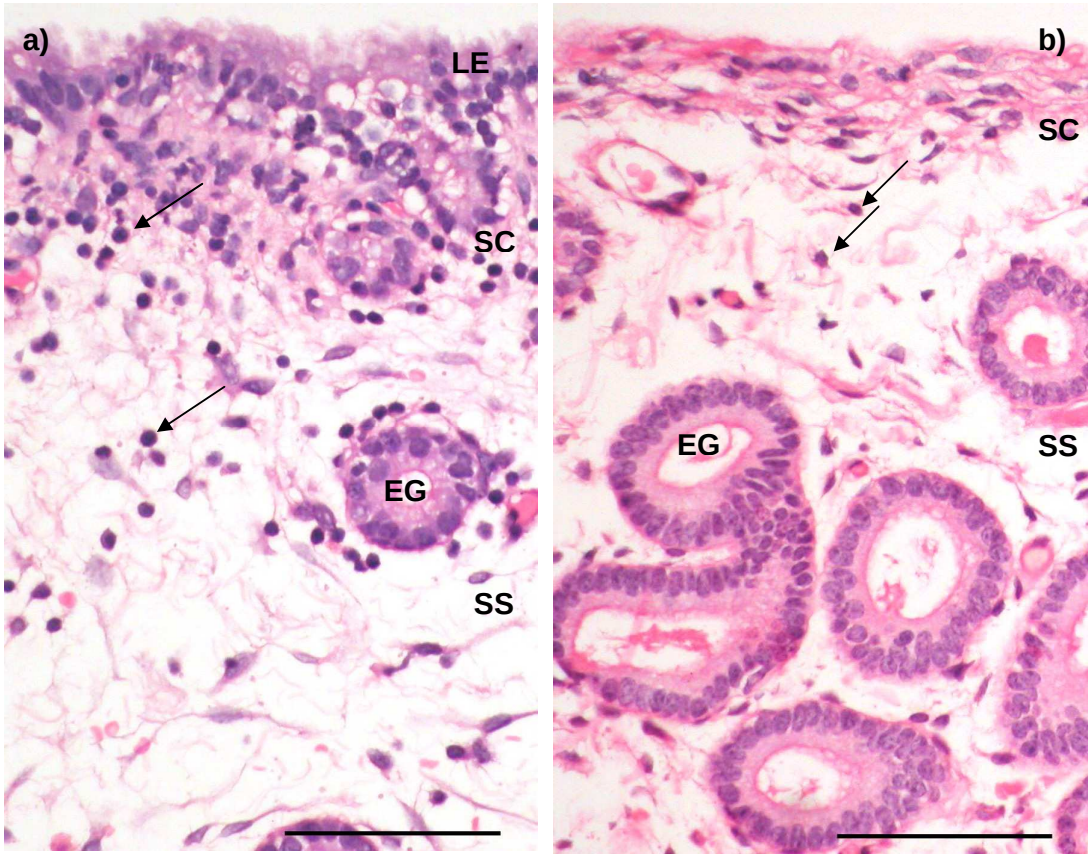


Figure 3.3 Control endometrial biopsies collected from mares before induction of endometritis. a) control sample taken in oestrus demonstrating tall columnar to pseudostratified luminal epithelium, loose stroma and low glandular density due to oedema, and widely scattered lymphocytes (arrows) in stratum compactum and stratum spongiosum. b) control sample taken in dioestrus demonstrating denser stroma and higher glandular density than in a) due to the absence of oedema. Artefactual damage has led to the loss of luminal epithelium. The stratum compactum can be seen to contain few lymphocytes, and some scattered lymphocytes (arrows) are seen in stratum spongiosum. EG: endometrial gland, LE: luminal epithelium, SC: stratum compactum, SS: stratum spongiosum. Samples were stained with H&E, scale bar represents 50µm.

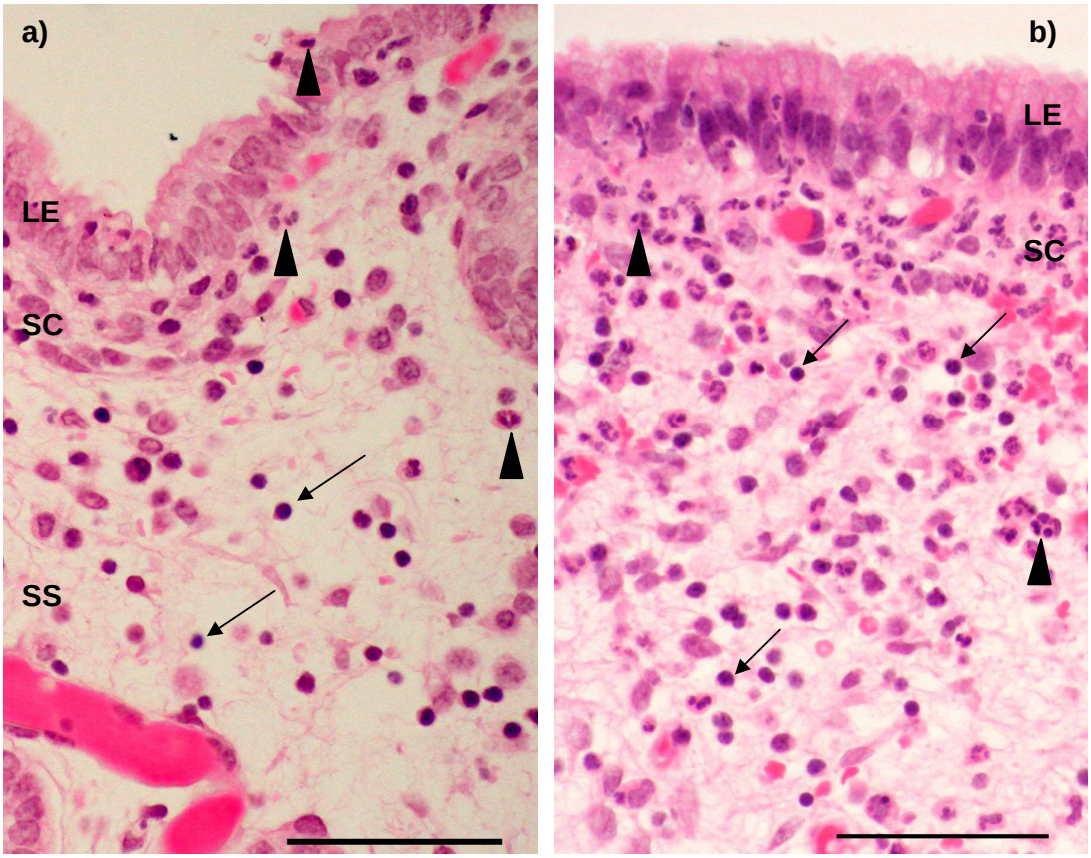


Figure 3.4 Endometrial biopsies collected from mares 20 hours after uterine inoculation with *S. zooepidemicus*. a) biopsy collected from mare 5 during streptococcal endometritis in oestrus demonstrating frequently scattered lymphocytes (arrows) and scattered neutrophils (arrowheads) in stratum compactum and stratum spongiosum. Neutrophils are also seen migrating through the luminal epithelium. b) biopsy collected from mare 1 during streptococcal endometritis in dioestrus demonstrating lymphocytes (arrows) and neutrophils (arrowheads) in stratum compactum and stratum spongiosum. LE: luminal epithelium, SC: stratum compactum, SS: stratum spongiosum. Samples were stained with H&E, scale bar represents 50µm.

In breeding-induced endometritis (**Figure 3.5**) the inflammatory cell infiltrate was generally milder than during the streptococcal-induced endometritis in oestrus and in dioestrus, consisting of fewer lymphocytes but more neutrophils. Neutrophils were present in all endometritis samples, both within tissue sections and as an exudate in the uterine and glandular lumina. No eosinophils were identified in the luminal epithelium in any model, but they were present in the stratum compactum and/or stratum spongiosum in all mares in all induced models. Widely scattered plasma

cells were present in most samples, mostly in stratum compactum and stratum spongiosum.

3.2.3 Quantitative analysis of luminal epithelium

Ten fields of luminal epithelium were examined in each biopsy from each mare, except for five fields in one dioestrus control biopsy and nine fields in one biopsy from streptococcal endometritis in oestrus due to artefactual absence of epithelium, see **Figure 3.3 b**. Numbers of lymphocytes were statistically significantly ($p=0.005$) higher in control samples during oestrus than during dioestrus. There was a statistically significant ($p=0.004$) increase in lymphocyte numbers during streptococcal endometritis in oestrus and dioestrus, as well as after breeding at oestrus, when compared to controls. The numbers of neutrophils increased in streptococcal endometritis, both in oestrus and dioestrus but no neutrophils were present in control samples. Plasma cells and eosinophils were only occasionally seen in LE.

3.2.4 Quantitative analysis of stratum compactum

In control samples numbers of lymphocytes were statistically significantly higher ($p=0.005$) in the stratum compactum during oestrus than during dioestrus (**Figure 3.6 a**). There was a statistically significant increase in lymphocyte numbers during streptococcal endometritis in oestrus ($p<0.001$) and in dioestrus ($p<0.001$) when compared to the controls. There was no statistically significant difference ($p=0.099$) in lymphocyte numbers after breeding when compared to oestrus controls. Lymphocyte numbers were statistically significantly lower after breeding than during

streptococcal endometritis in oestrus ($p<0.001$) and dioestrus ($p<0.001$). No statistically significant difference ($p=0.941$) was found in lymphocyte numbers between oestrus and dioestrus during streptococcal endometritis.

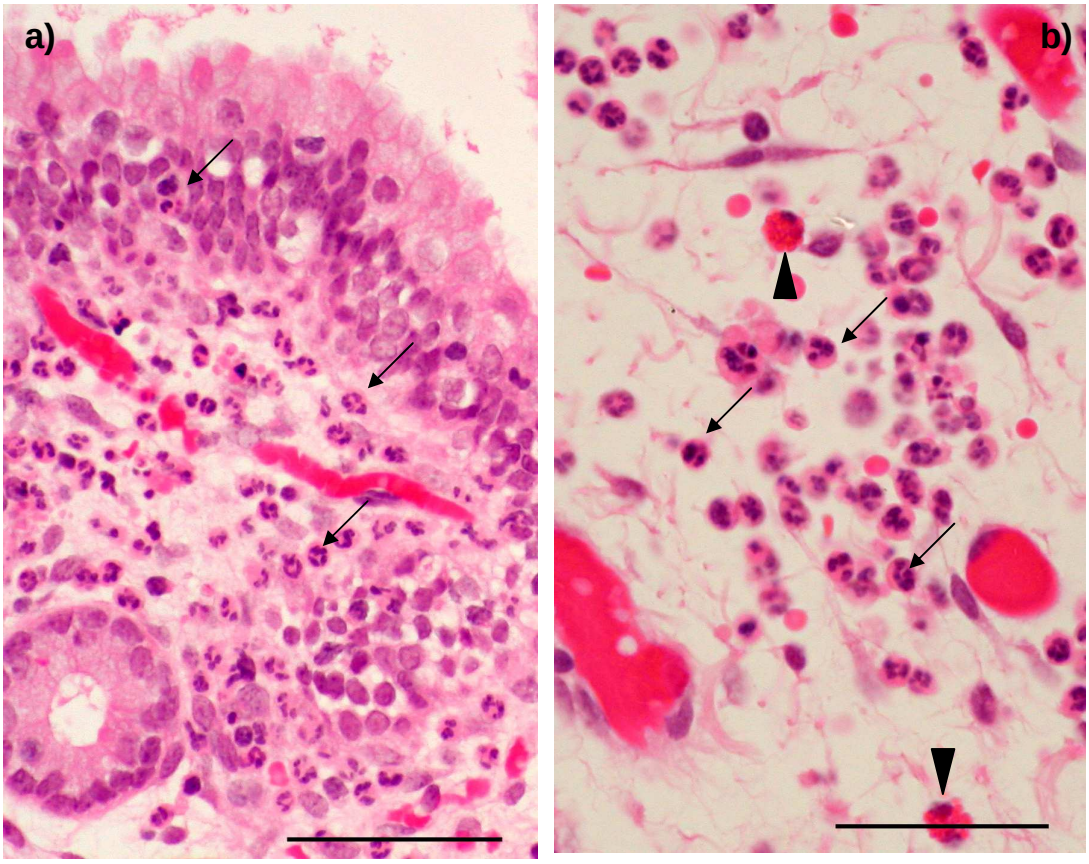


Figure 3.5 Endometrial biopsies collected from a mare 20 hours after natural breeding. a) high power (400X; scale bar: 50µm) magnification demonstrating neutrophil infiltration in stratum compactum, and stratum spongiosum, as well as neutrophil transmigration through luminal epithelium. b) high power (600X; scale bar: 25µm) magnification of stratum spongiosum demonstrating frequently scattered neutrophils (arrows) and scattered eosinophils (arrowheads) LE: luminal epithelium, SC: stratum compactum, SS: stratum spongiosum. Samples were stained with H&E.

There was an increase in neutrophil numbers in the stratum compactum in all three induced endometritis models (i.e. bacterial inoculation in oestrus, bacterial inoculation in dioestrus and breeding induced endometritis; **Figure 3.6 c**).

An overwhelming majority of the fields analysed in oestrus and dioestrus control samples contained no neutrophils, although the occasional cell was observed. The increase seen during streptococcal endometritis in dioestrus was statistically significant ($p<0.001$) as some of the control fields had contained the occasional cell, but was significantly ($p<0.001$) smaller than during streptococcal endometritis in oestrus. Neutrophil numbers after breeding were statistically significantly ($p<0.001$) higher than during streptococcal endometritis during dioestrus but did not differ ($p=0.113$) from streptococcal endometritis in oestrus.

Increased eosinophil numbers were seen in all three types of induced endometritis (**Figure 3.6 e**). When compared with controls, eosinophil numbers in streptococcal endometritis during oestrus ($p=0.210$) and dioestrus ($p=1.000$) and in breeding-induced endometritis ($p=0.652$) were not statistically significant. The greatest increase in eosinophil numbers occurred in streptococcal endometritis in dioestrus, that was statistically significantly ($p<0.001$) greater than in the other types of endometritis. The occasional plasma cell was seen in all samples, with no statistically significant difference in numbers between controls and induced endometritis samples (**Figure 3.6 g**).

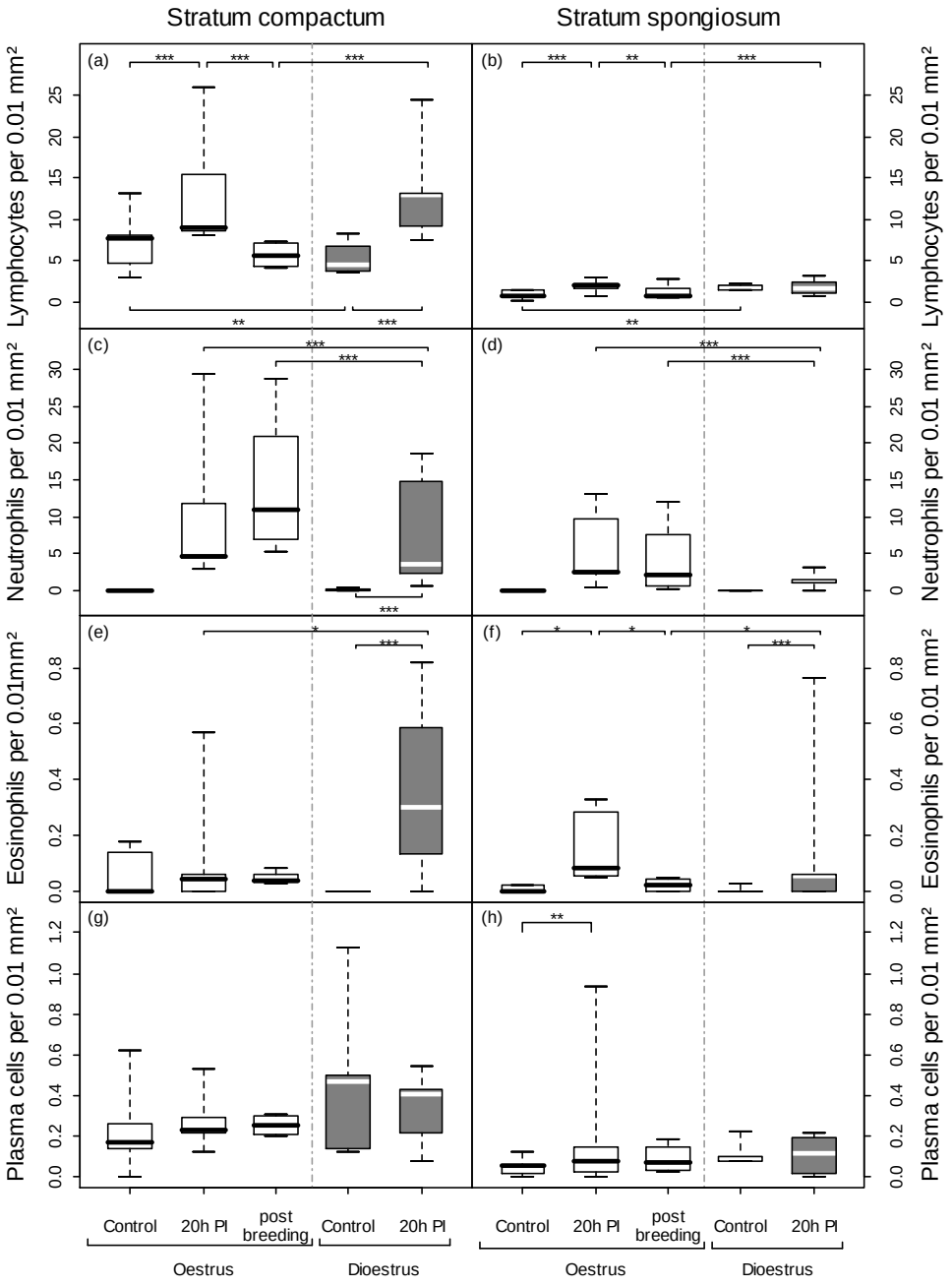


Figure 3.6 Boxplot illustrating numbers of endometrial inflammatory cells. Numbers of lymphocytes, neutrophils, eosinophils and plasma cells per 0.01 mm^2 in equine endometrium are shown before and 20 hours after induction of experimental endometritis in oestrus and dioestrus. Control: samples collected before induction of endometritis. 20h PI: 20 hours after uterine inoculation with *S. zooepidemicus*. Post breeding: 20 hours after natural breeding to a stallion. *: p<0.01, **: p<0.05, ***: p<0.001.

3.2.5 Quantitative analysis of stratum spongiosum

Lymphocyte numbers in control samples were statistically significantly ($p<0.005$) lower during oestrus than during dioestrus (**Figure 3.6 b**). During induced streptococcal endometritis in oestrus there was a statistically significant ($p<0.001$) increase in lymphocyte numbers. The increase seen in streptococcal endometritis in dioestrus was not statistically significant ($p=0.991$), as numbers in control samples had been relatively high and variation between mares was greater. No statistically significant difference ($p=0.294$) was demonstrated after breeding in oestrus, and lymphocyte numbers were statistically significantly lower than in streptococcal endometritis in oestrus ($p=0.003$) and dioestrus ($p=0.009$).

In control biopsies, no neutrophils were seen in the stratum spongiosum, but great numbers of neutrophils were observed in all induction models (streptococcal endometritis, both in oestrus and dioestrus and breeding-induced endometritis; **Figure 3.6 d**). Neutrophil numbers during streptococcal endometritis in dioestrus were statistically significantly lower than during streptococcal endometritis in oestrus ($p<0.001$) and after breeding ($p<0.001$).

The occasional eosinophil was seen in control samples, but there was no statistically significant difference ($p=0.845$) between oestrus and dioestrus (**Figure 3.6 f**). A statistically significant increase in eosinophil numbers occurred during streptococcal endometritis in oestrus ($p=0.012$) and dioestrus ($p<0.001$). No statistically significant difference ($p=0.166$) was seen in eosinophil numbers after breeding when compared to oestrus controls. The increase in eosinophil numbers was statistically

significantly larger during streptococcal endometritis in oestrus (p=0.016) and dioestrus (p=0.019) than during breeding-induced endometritis. The occasional plasma cell was seen in control samples, and there was a tendency (p=0.059) for higher numbers during dioestrus (**Figure 3.6 h**). During streptococcal endometritis in oestrus there was a statistically significant increase (p=0.014) in plasma cell numbers when compared to oestrus control, due to very large numbers in only one of the fields analysed. No statistically significant increases were seen in streptococcal endometritis during dioestrus (p=0.498) and during breeding-induced endometritis (p=0.115).

3.3 Discussion

This study has quantified for the first time the density of cellular infiltrations that occur in the equine endometrium during two distinct stages of the oestrous cycle and during induced endometritis. Studies have been carried out previously by counting inflammatory cells in the endometrium, but have not included both stages of the oestrous cycle or made comparisons before and after induction of endometritis. Small numbers of randomly distributed lymphocytes were observed in the stratum compactum of dioestrous mares by Keenan and coworkers (1987), who also found eosinophil numbers to be decreased with increasing progesterone concentrations, but no oestrous mares were sampled to enable comparisons between the stages of the oestrous cycle (Keenan *et al.* 1987). In a study on oestrous mares, endometrial biopsies stained with H&E were analysed and various factors compared between normal mares and mares with reproductive problems. This study divided infiltrating cells into segmented cells (neutrophils and eosinophils) and mononuclear cells

(lymphocytes, plasma cells, monocytes/macrophages and mast cells) but did not distinguish them further (Leishman *et al.* 1982). No difference was demonstrated in the degree of mononuclear infiltration between normal and barren mares (Leishman *et al.* 1982).

In the present study computer-assisted counting methods were used to quantify four types of inflammatory cells of interest in equine endometritis, easily identified in H&E staining. Other cells such as mast cells and macrophages require specific staining methods or immunohistochemistry for positive identification (Tekin & Hansen 2004; Sugamata *et al.* 2005). Macrophages would be particularly interesting within the focus of this study due to their activating role of myofibroblasts in collagen remodelling as demonstrated in pathogenesis of liver fibrosis (reviewed by Bataller & Brenner 2005). Macrophages containing hemosiderin (siderocytes) can be positively identified in H&E sections (Kenney 1978), however macrophages not containing hemosiderin are likely to be overlooked. In a previous study on oestrous equine endometrial biopsies, siderocytes tended to be less prominent in mares that had been barren 2-3 years than in mares that were not barren or had been barren 4 or more years (Leishman *et al.* 1982). The markers required for positive identification of macrophages were not available in this laboratory and it was decided due to temporal and financial constraints not to include these cells in the analysis. However, this would be a valuable focus for future study of the pathogenesis of equine endometrial fibrosis.

Clinical observations were made before samples were collected in order to correlate these with the histological description of endometrial biopsies. The clinical response, including uterine oedema and intrauterine fluid tended to be more severe to streptococcal inoculation than to breeding. It is clear that individual variation in clinical and histological signs of endometritis is considerable, and that uterine oedema as diagnosed by transrectal ultrasonography is invariably confirmed when histological analysis is carried out. However, there was a tendency for the subjective scoring system of uterine oedema to yield consistently higher scores when judged by histological sections than by transrectal ultrasonography. This may be explained by the fact that histology is a more sensitive method of diagnosing stromal oedema, and by this method it is possible to detect discreet areas where stromal oedema is greater than in the remainder of the section. In their study of endometrial sections taken from oestrous mares, Leishman *et al.* (1982) found great variation in oestrous cycle-dependent stromal oedema within and between individuals. Although this type of study would benefit from a larger sample population due to the great individual variation in clinical and histological response, the present study did demonstrate statistically significant differences in cellular infiltrations, not only between endometritis models and their controls, but also between different endometritis models.

Biopsies were collected at 20 hours after induction of experimental endometritis, and although it would have been informative to collect biopsies earlier on, it was decided to minimize endometrial injury until uterine lavages had been collected due to the inducing effect this would invariably have on the activity of MMPs in endometrial

secretions. The study was carried out on a small population sample and can therefore only be used as a guide to the histology of acute endometritis. The clinical response displayed by the mares showed great individual variation, indicating that a larger sample would benefit this type of study. Although the mares were monitored clinically to observe the timeline of recovery, endometrial biopsies were not collected once clinical signs of endometritis had resolved. This was done to minimize the need for transcervical manipulation of the uterus, that can lead to endometritis (Williamson *et al.* 1987; McDonnell & Watson 1992) although it would have been interesting to observe the density of each cell type after recovery.

Lymphocytic infiltrations have not previously been demonstrated to vary with the stage of the oestrous cycle, and they have been attributed to chronic rather than acute endometritis (Kenney 1978). In this study, they were found to react as part of the acute inflammatory response within 20 hours after induction of endometritis, and their reaction to streptococci was more intense than to semen. Lymphocytes play a central role in the uterine adaptive immune system (reviewed by Brandtzaeg 1997) and usually appear somewhat later than neutrophils in most inflammatory reactions. However, human endometrial lymphocytes attract neutrophils by inducing the secretion of cytokines from stromal fibroblasts (Kelly *et al.* 2001). The results presented here indicate that lymphocytes appear together with neutrophils within the first 20 hours after induction of equine endometritis, especially during *S. zooepidemicus* infection. It is likely that the presence of bacterial antigens induces a sustained attraction of lymphocytes that in turn increases the chemotaxis of neutrophils to the uterus. Previous studies have indicated that the adaptive immune

response involving T-lymphocytes takes place within the acute inflammatory response at least in acute semen-induced equine endometritis (Tunon *et al.* 2000). Therefore it would seem that the immediate immune response in the uterus not only involves granulocytes, but also lymphoid cells.

In the present study semen was introduced into the uterus by natural mating, so could have contained up to 6 x 10^5 cfu/ml of mucosal microorganisms, including *S. zooepidemicus* (Simpson *et al.* 1975). The ejaculate is likely to have contained minimal numbers of bacteria, as microbiological cultures of swabs taken from the stallion reproductive tract revealed no pathogens and very limited growth of other microorganisms. Lymphocyte density increased less severely after breeding than after uterine inoculation with streptococci, and this effect is likely to be due to the specific antigen-provoked inflammatory response to streptococci. The immunomodulatory effect of seminal plasma resulting from the reduction in neutrophil-sperm binding could also have the wider effect of inhibiting lymphocyte activation and opsonin secretion, subsequently reducing phagocytosis of bacteria as well as sperm.

According to this study, neutrophil density depended more on the stage of the cycle than on the type of inflammatory stimulus. Higher numbers of neutrophils were present during both types of endometritis in oestrus (streptococcal and breeding-induced) than during streptococcal endometritis in dioestrus. This implies that uterine neutrophils are preferentially recruited under the influence of oestrogen, as has been demonstrated in the human (Salamonsen *et al.* 2002). Kenney (1978)

reported that neutrophils were more prominent in the equine endometrium during oestrus, demonstrated by margination of these cells in endometrial capillaries. In addition, other studies demonstrated less phagocytic activity of neutrophils *in vitro* under the influence of progesterone (Ganjam *et al.* 1982; Asbury & Hansen 1987). No difference was observed in neutrophil density during breeding-induced and streptococcal endometritis in oestrus. However, it is known that seminal plasma has an inhibitory effect on neutrophil phagocytosis by blocking the sperm-neutrophil binding *in vitro* (Alghamdi *et al.* 2004) and therefore less phagocytic activity could be expected following breeding.

In this study eosinophils were seen in varying numbers in the normal endometrium. Eosinophils play a role in tissue remodelling and healing of the human endometrium (Blumenthal *et al.* 2000) and their recruitment is induced by oestradiol (Leiva *et al.* 1991). They have been observed more frequently during equine endometrium in oestrus, and associated with pneumovagina, possibly due to an undefined antigen-antibody reaction (Kenney 1978; Slusher *et al.* 1984). In the present study there was no cyclical correlation in eosinophil density, but as the mares all had normal perineal conformation and no signs of pneumovagina, an increased infiltration of eosinophils were not expected in control sections. An increase in eosinophil density was seen in the stratum compactum and stratum spongiosum following endometritis and was associated more with streptococcal than breeding-induced endometritis. A longer standing uterine irritation would be expected to lead to a more pronounced increase in eosinophil numbers as this cell type has been associated with more established inflammation of the equine endometrium (Olsen *et al.* 1992).

Plasma cells have been associated with chronic endometritis, most often in the upper stratum compactum although they can be found anywhere in the endometrium, and have been reported in the endometrium 3-4 weeks following experimental inoculation with *S. zooepidemicus* (Ganjam *et al.* 1982). They are the major producer of antibody, and their presence indicates the continuing presence of antigen, presumed to be microbial in nature. In an experimental study on contagious equine metritis the inflammation was predominantly plasmacytic 14 days post-inoculation, after which numbers of plasma cells decreased and the prevailing inflammation became primarily lymphocytic (Acland & Kenney 1983). The present study was carried out within the first hours after introducing inflammatory stimulus and therefore a difference in plasma cell density was not expected. The statistically significant increase in plasma cell numbers demonstrated in endometritis during oestrus was due to atypically high numbers in one of the analysed fields.

3.4 Conclusion

This study has demonstrated that the reaction of uterine lymphocytes and eosinophils appears to depend on the type of inflammatory stimulus rather than on the stage of the oestrous cycle. The reaction of endometrial neutrophils depends more on the stage of the oestrous cycle, as they react in lower numbers to streptococcal infection in dioestrus than in oestrus. It is possible that the immunomodulating effect of seminal plasma influences the recruitment and activation of lymphocytes as well as the phagocytosis by neutrophils. It has been demonstrated that an observation of lymphocytic infiltration in an endometrial biopsy is not necessarily an indication of chronic inflammation, as lymphocytic numbers are increased during acute

endometritis and during normal dioestrus in the absence of infection. If bred to a stallion with a high number of genital streptococci, a mare can be expected to exhibit considerable lymphocytic infiltrations, and these are likely to resolve once the infection is removed. Future studies should be carried out with a larger population sample, and should include an endometrial sample collected after recovery from endometritis as well as a count of macrophages in the endometrium.

4 Chapter 4:

Activity of MMP-2, MMP-9 and TIMPs during equine endometritis

4 Activity of MMP-2, MMP-9 and TIMPs during equine endometritis

The uterine remodelling taking place around menstruation in women involves the expression, secretion and activation of MMPs (reviewed by Salamonsen 2003). The activities of MMP-9 and -2 play a role in the formation of endometrial cups during equine pregnancy (Vagnoni *et al.* 1995) and MMP-2 has been identified around fibrotic glands of the non-pregnant equine endometrium (Walter *et al.* 2005). However, the secretion of these MMPs in the normal non-pregnant equine endometrium during the oestrous cycle, and during endometritis has not previously been investigated.

4.1 Materials and methods

4.1.1 Animals and sample collection

Five mares of various breeds, ranging in age from 4 to 12 years and weighing 430 to 700 kg were studied through four consecutive spontaneous oestrous cycles (the same animals as in chapter **3**). The mares were reproductively healthy as confirmed by clinical examination (see **2.1.2**), clitoral, vaginal and endometrial swabs (see **2.1.3**), and a histopathological assessment category I-IIA (Kenney and Doig 1986) of H&E stained sections of endometrial biopsies. Endometritis was induced in oestrus and dioestrus using uterine inoculation with *S. zooepidemicus* (see **2.2.2**) and in oestrus by mating four of the mares to a stallion (see **2.2.3**). One 14 year old crossbred stallion was used for natural mating, and was confirmed to be free from pathological genital infections by microbiological culture of penile swabs (see **2.1.4**).

Clinical findings were recorded as described in **2.1.2** before carrying out any manipulations of the reproductive tract at each stage of the protocol. Five hours after inducing endometritis, clinical findings were again recorded (chapter **3**) before collecting 60 ml uterine lavages (see **2.1.5**) and again 20 hours after inducing endometritis (chapter **3**), before collecting uterine lavages (see **2.1.5**) and endometrial biopsies (see **2.1.6**; frozen using OCT). After the collection of samples, mares were examined daily to monitor the progression of endometritis.

4.1.2 Sample analysis

The presence of MMP-2 and MMP-9 (gelatinases) in uterine lavage fluid was investigated using gelatin zymography (see **2.4.3**). Endometrial cryostat sections were examined for the presence of active forms of gelatinases by gelatin substrate *in situ* zymography as described in **2.5.1**. In order to identify neutrophils in endometrial cryostat sections in an attempt to colocalise them with gelatinase activity, neutrophil myeloperoxidase antibody was applied to consecutive sections subjected to the *in situ* zymography method (see **2.5.2**). The presence of TIMPs in uterine lavage fluid was detected using reverse zymography (see **2.4.4**).

4.1.3 Statistical analysis

Due to the each mare being subjected to the same endometritis induction model in oestrus and dioestrus (paired repeated measures), generalised linear mixed effect models were used for statistical analysis (Pinheiro & Bates 2000). The animal from which the uterine lavage fluid was taken was entered into the models as a random effect. The stage of each endometritis induction model (control, during

streptococcal endometritis and during breeding-induced endometritis) and the stage of the oestrous cycle (oestrus or dioestrus) were entered as the fixed effect. Results were considered to be statistically significant when $p<0.05$.

4.2 Results

4.2.1 Detection of MMP-2 and MMP-9 by gelatin zymography

Gelatin zymography detected MMP-2 and MMP-9 in uterine lavage fluid (**Figure 4.1**). The source of these bands was confirmed to be metalloproteinase activity by the total inhibition of gelatinase activity by EDTA and 1,10-phenanthroline (see **1.3.1.1**). Latent (92 kDa) and active MMP-9 (86 kDa) were detected but only the latent form of MMP-2 (72 kDa) was present. The quantification of MMP activity by gelatin zymography is limited by the fact that densitometric analysis can be accurate only at picogram levels of MMPs when bands are very faint and distinct.

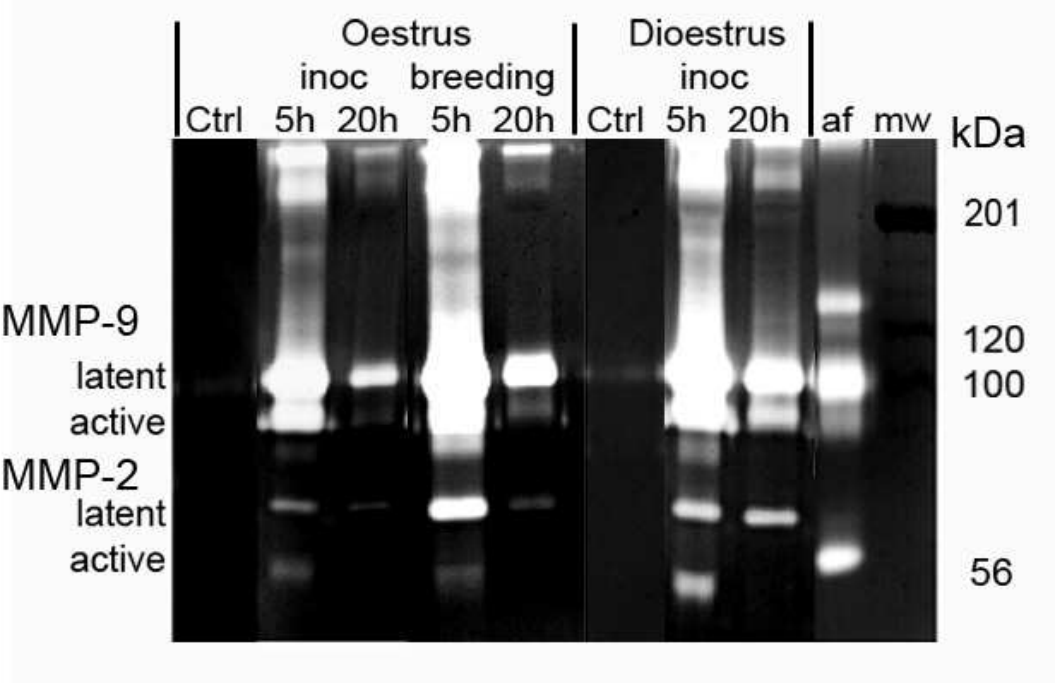


Figure 4.1 Zymogram of MMP-9 and MMP-2 activities in equine uterine fluid. Representative gelatin zymography of uterine lavage samples from mares during normal oestrus and dioestrus (Ctrl=control), and 5 and 20 hours after *S. zooepidemicus* inoculation (inoc) in oestrus and dioestrus, as well as 5 and 20 hours after breeding in oestrus. For characterization of MMPs was human amniotic fluid (af), mw: Molecular mass markers.

Basal levels of latent MMP-9 (**Figure 4.2 a**) and latent MMP-2 (**Figure 4.2 b**) in control samples did not differ significantly between oestrus and dioestrus. The activities of latent and active MMP-9, and latent MMP-2 were dramatically increased after *S. zooepidemicus* inoculations (oestrus and dioestrus) and after breeding when compared to basal levels ($p<0.001$ in all cases).

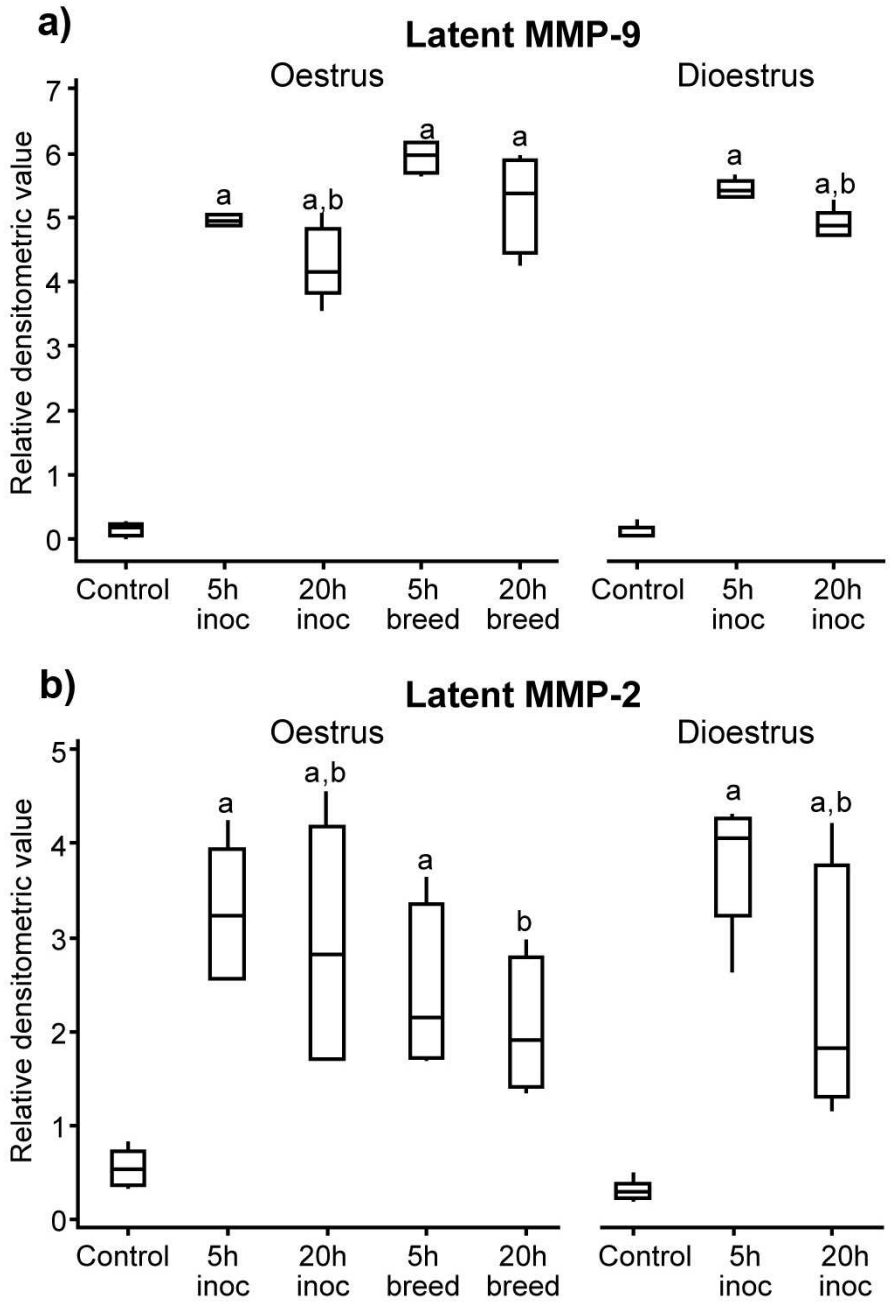


Figure 4.2 Boxplots illustrating MMP-9 and MMP-2 activity in uterine fluid. Relative densitometric values and standard deviation of latent MMP-9 a) and latent MMP-2 b) in equine uterine lavages as detected by gelatin zymography. [a]significantly different ($p<0.0001$) from corresponding control, [b]significantly different ($p<0.05$) from corresponding sample collected 5 hours after induction of endometritis. Samples taken before (control), and 5 and 20h after induction of experimental endometritis (inoc: inoculation with *S. zooepidemicus*, breed: natural breeding to a stallion).

Bands corresponding to active MMP-9 were identified in many samples, but this form of the enzyme is unstable and highly variable so was not analysed quantitatively. The activity of latent MMP-9 was more profoundly induced during endometritis than the activity of latent MMP-2 and therefore serial dilutions of uterine lavage samples were carried out in order to more accurately quantify the activity of latent MMP-9. Additionally, less variation was observed in latent MMP-9 activity between individual animals than in latent MMP-2 activity. Measured activity of latent MMP-9 five hours after uterine streptococcal inoculation in oestrus was statistically significantly lower than five hours after inoculation in dioestrus ($p<0.001$) and five hours after breeding ($p=0.011$). Between five and 20 hours after induction of streptococcal endometritis there was a statistically significant decrease in latent MMP-9 expression in oestrus ($p=0.040$) and dioestrus ($p=0.008$) but no statistically significant change was seen between five and 20 hours after breeding ($p=0.206$).

4.2.2 Localization of gelatinase activity by *in situ* zymography

In situ zymography revealed distinct patterns of gelatinase activity in endometrial sections, especially in stratum spongiosum in discrete granules either within cells in the stroma or extracellularly (**Figure 4.3 a**). Only occasional cells exhibited gelatinase activity in control samples, and an increase in the numbers of these cells was seen during endometritis. Fluorescence, as a measure of gelatinase activity was inhibited by 1,10-phenanthroline, showing that the gelatinase activity displayed by these cells was of metalloproteinase origin. Negative controls verified the absence of intrinsic fluorescence (**Figure 4.3 b**). However, the specific inhibitor

of MMP-2 did not demonstrate any inhibiting effect on gelatinase activity in the tissue. The specific inhibitor of MMP-9 had a slight inhibitory effect at the concentrations of 5nM and 5µM but did noticeably decrease the gelatinase activity at 50nM.

The gelatinase-positive cells were identified according to histological criteria as infiltrating inflammatory cells with the characteristics of eosinophils, being approximately 10-12µm in diameter with multilobular nuclei and an abundance of large cytoplasmic granules. Nevertheless, neutrophils in endometrial sections taken during endometritis were more numerous (see chapter **3**), and therefore it was plausible that some MMP-9 activity was localised in neutrophils. To distinguish between neutrophils and eosinophils in determining the cellular localization of gelatinase activity, immunolocalization of MPO was undertaken. Very few MPO positive cells were detected in control samples, but in samples taken during endometritis large numbers of MPO-containing neutrophils were identified immediately below the luminal epithelium (**Figure 4.3 c**). Negative controls verified the absence of non-specific antigen-antibody reactions.

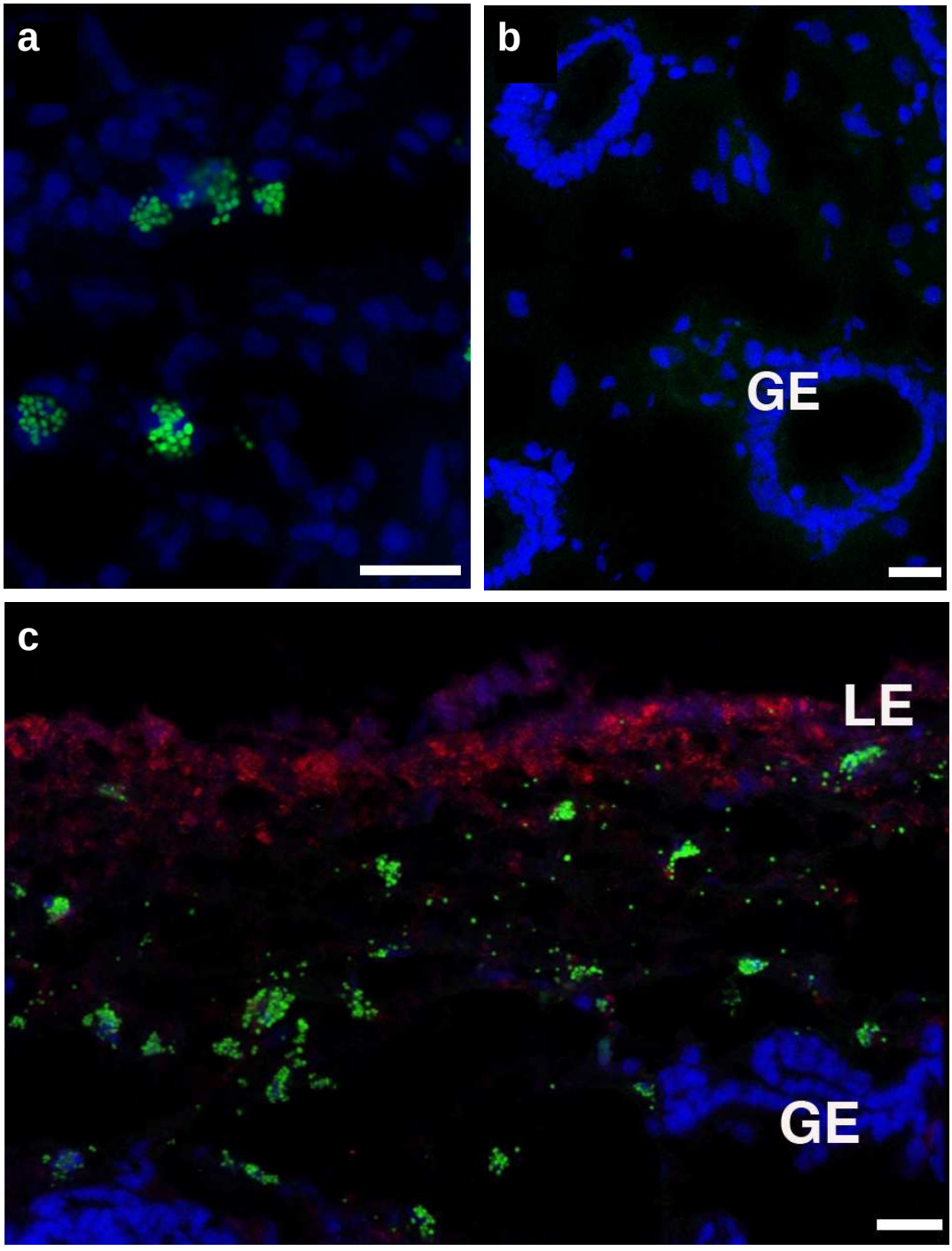

a
b
GE
c
LE
GE

Figure 4.3 (previous page). Photomicrographs of gelatinase activity in inflammatory cells in the equine endometrium a) Confocal photomicrograph of equine endometrial sample from mare 1, demonstrating infiltrating inflammatory cells containing fluorescent green cytoplasmic granules indicating gelatinase activity in stratum spongiosum during endometritis in oestrus. b) Negative control sample obtained from the endometrium of mare 1 during oestrus, exhibiting no autofluorescence. c) Equine endometrium collected from mare 3 during endometritis in dioestrus showing neutrophils containing myeloperoxidase (fluorescent red stain) below and within the luminal epithelium and infiltrating inflammatory cells in stratum spongiosum containing green fluorescing cytoplasmic granules, indicating gelatinase activity. Blue nuclear stain: DAPI. LE: luminal epithelium, GE: glandular epithelium. Scale bars represent 20µm.

Colocalization of MPO and gelatinase activity was not observed, further confirming that eosinophils were the source of active gelatinase granules detected by *in situ* zymography.

4.2.3 Detection of TIMP activity by reverse zymography

Reverse zymograms revealed the presence of TIMP-1 and -2, and the glycosylated and unglycosylated forms of TIMP-3 in all uterine lavages (**Figure 4.4 a**). The activity of TIMP-2 was consistently and statistically significantly higher ($p=0.015$; **Figure 4.4 b**) in dioestrus control samples than at oestrus in all mares. The activity in samples taken during endometritis tended to be lower than in control samples, with a statistically significant ($p=0.007$) reduction in TIMP-2 activity demonstrated five hours post-inoculation in dioestrus. The activities of TIMPs -1 and -3 were low and no changes were detected.

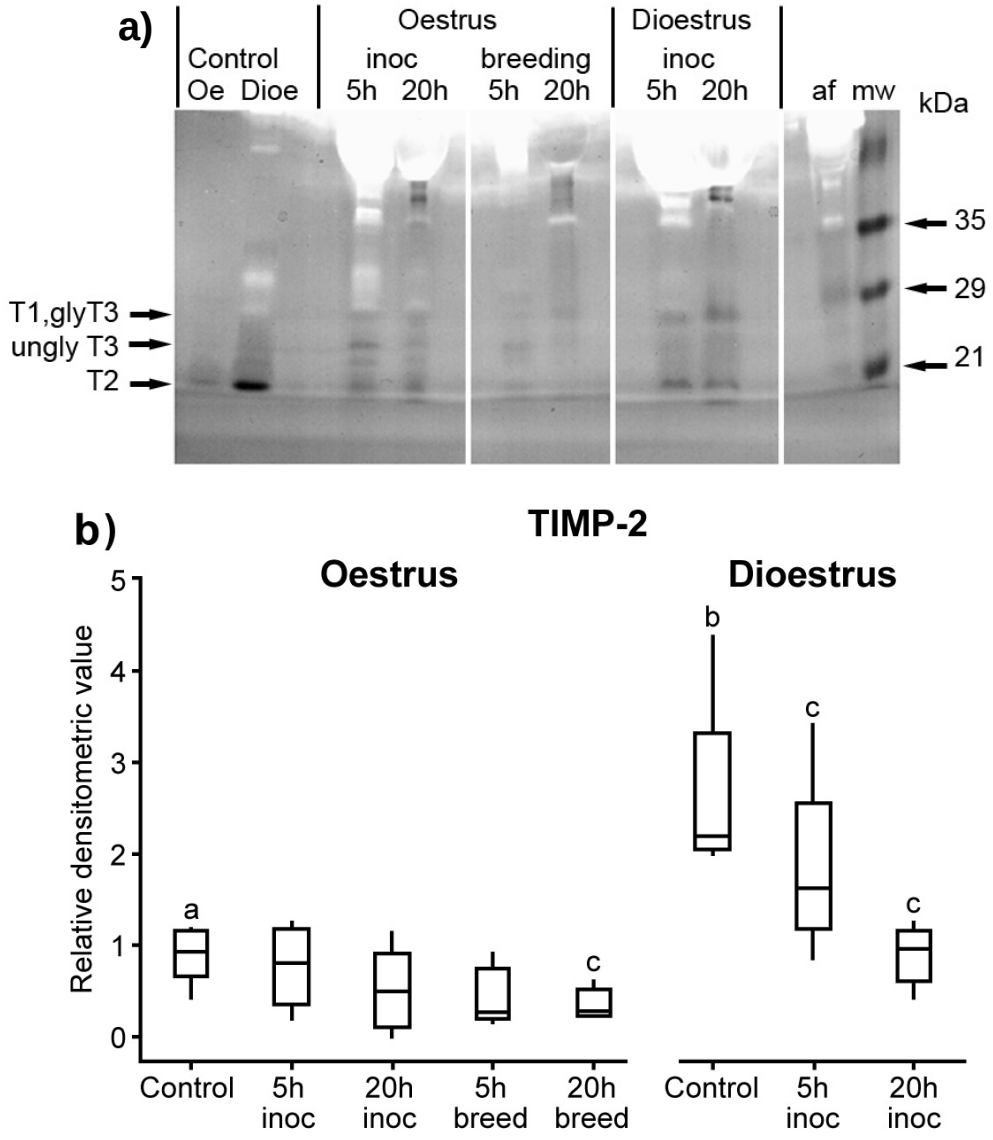


Figure 4.4 TIMP activities in equine uterine fluid. a) Representative reverse zymography gel of uterine lavages from mares during normal oestrus and dioestrus (Control), 5 and 20 hours after *S. zooepidemicus* inoculation (inoc) in oestrus and dioestrus, and 5 and 20 hours after natural breeding. TIMP activity is visualised as three darker bands of 27-30 kDa (corresponding to the molecular masses of TIMP-1, glycosylated TIMP-3 and TIMP-4), 24 kDa (unglycosylated TIMP-3) and 21 kDa (TIMP-2). For characterization of TIMPs was human amniotic fluid (af), mw: Molecular mass markers. b) Boxplot illustrating the relative densitometric values and standard deviation of TIMP-2 as detected in uterine flushes by reverse zymography. [a] and [b]: significantly different ($p<0.0001$), [c]significantly different ($p<0.05$) from corresponding control. breed: natural breeding.

4.3 Discussion

In this study it has been shown for the first time that the gelatinases MMP-2 and MMP-9 are induced during both bacterial and breeding-induced equine endometritis. This suggests that they are involved in the tissue remodelling and repair process following an inflammatory response in the endometrium. Moreover, this is the first time that TIMP activities have been reported in equine uterine fluid.

The method of gelatin zymography is reliable and well documented on fluid samples collected from equine ovarian follicles (Riley *et al.* 2004) and synovial joints (Clegg *et al.* 1997b), as well as equine urine (Arosalo *et al.* 2007). Reverse zymography has previously demonstrated TIMPs in equine ovarian follicular fluid (Riley *et al.* 2001) and this method is therefore considered reliable in the detection of equine TIMPs.

Whereas the present study was conducted on uterine lavages most studies on human endometrial MMP expression using gelatin zymography have been conducted on homogenised endometrial tissue (Tamakoshi *et al.* 1994) or supernatant from cultured endometrial cells (Salamonsen *et al.* 1997; Mulayim *et al.* 2004). However, uterine lavages have been reported to accurately correlate the activity of MMP-2 and MMP-9 in human endometrial cancer, validating the use of uterine lavage fluid to assess the endometrial expression of these enzymes (Lopata *et al.* 2003).

In situ zymography does not use agents such as SDS which open up the active site of latent forms to demonstrate gelatinase activity in gelatin zymography, and therefore exclusively demonstrates the active forms of MMP-2 and MMP-9. The fluorescence

exhibited in the stratum spongiosum of endometrial biopsies is therefore an expression of native gelatinase activity. This activity was verified to be due to MMPs, specifically MMP-9, using generic and gelatinase specific inhibitors.

It was demonstrated that during normal early oestrus and mid-dioestrus the activity of MMP-2 and MMP-9 in uterine secretions is low and therefore they are unlikely to be required during the normal equine oestrous cycle. At this early stage of oestrus, high concentrations of oestrogen and low concentrations of progesterone coincide, whereas during mid-dioestrus the oestrogen:progesterone ratio is at a minimum (Daels & Hughes 1993). In human endometrium, the fall in progesterone concentration preceding menstruation induces an increased expression of MMP-2 and MMP-9 (reviewed by Henriet *et al.* 2002). In the present study the basal secretion of MMP-2 and MMP-9 into the uterine lumen was low and no difference was observed depending on the stage of the oestrous cycle. In women, MMP-2 expression in the endometrium is constitutive with an increase around menstruation, and MMP-9 expression is minimal apart from during menstruation (Goffin *et al.* 2003). The fact that such cyclical variation was not demonstrated in the mare is unsurprising as the endometrium does not undergo the same degree of remodelling in the equine oestrous cycle as occurs during the menstrual cycle in women. However, it was demonstrated that the inhibitory activity of TIMP-2 is higher during dioestrus than during oestrus. By contrast, studies on human endometrium report that TIMPs remain relatively unaltered through the cycle (Goffin *et al.* 2003). It was surprising that TIMP-2 was induced during dioestrus in the relatively quiescent equine endometrium. The expression of TIMP-2 tended to decrease at the same time as an

increase was seen in MMP-9 and -2 activities after endometritis was induced. This also shows a different pattern of regulation to the human endometrium, where a fluctuation in MMPs is not met by a fluctuation in TIMP-2 activity during the inflammation-like stage of menstruation (Goffin *et al.* 2003). The effect of TIMP-2 is biphasic, in that it is important in the activation of MMP-2 but inhibits it at higher levels. The increase in TIMP-2 levels during dioestrus may be specifically aimed at inhibiting MMP-2 activity, but conversely the decreased levels of TIMP-2 during endometritis are able to selectively activate MMP-2 (Strongin *et al.* 1995). However, it is possible that this decrease is a result of a dilution of uterine contents by transudate, and that the secretion of TIMP-2 is indeed unchanged in endometritis.

In this study the response to uterine insult by induction of MMP secretion was rapid and especially striking for latent MMP-9. The reaction in latent MMP-9 activity five hours after induction of experimental endometritis appeared controlled due to a relatively limited individual variation. In uterine lavages collected 20 hours after induction of endometritis the activity of latent MMP-9 had begun to subside, and the variation in latent MMP-9 activity between mares had increased. This indicates that the acute inflammatory response involving leukocytes and activating factors in mares is relatively homogeneous but as the response develops it becomes increasingly heterogeneous. The response of latent MMP-2 was also acute, as activities were already subsiding by 20 hours after induction of endometritis. Individual variation in latent MMP-2 activity was relatively great, and as already mentioned, the activity was lower than latent MMP-9 activity in corresponding concentrations. It is also possible that increasing amounts of transudate in the uterine lumen at 20 hours after

induction of endometritis as demonstrated in **chapter 3** contributed to a dilution of the MMPs already secreted into the uterine lumen. It is plausible, however that additional amounts of MMP-9 are not being secreted at this time after the induction of endometritis.

The inflammatory response following *S. zooepidemicus* inoculation at oestrus provoked the least increase in latent MMP-9 activity. This is surprising as uterine inflammatory mechanisms including neutrophil activity (Watson *et al.* 1987b; Watson & Stokes 1988) and immunological function of uterine fluid (Blue *et al.* 1982) are more efficient under the influence of oestrogen than under high concentrations of progesterone. However, the most profoundly increased activity of latent MMP-9 was observed 5 hours after breeding in oestrus. Natural breeding introduces sperm as well as potentially pathogenic surface microorganisms into the uterus that might amount to an additive effect on MMP activity by these two inflammatory stimuli. This has not been shown to be the case when observing the clinical response following natural breeding and AI (Nikolakopoulos & Watson 1997; Katila 2005). Furthermore, it is known that although spermatozoa induce an inflammatory response in the uterus through compliment activation (Troedsson *et al.* 2001) this is counteracted by an immunosuppressive effect on uterine neutrophils by seminal plasma (Alghamdi *et al.* 2004). In the present study, seminal plasma did not suppress the activity of MMP-9 in the presence of spermatozoa and microorganisms although an immunosuppressive effect on neutrophil phagocytic activity is likely to have been present (Nikolakopoulos & Watson 1997; Alghamdi *et al.* 2004;).

Gelatinase activity was demonstrated in the cytoplasm of infiltrating eosinophils in the endometrial stroma. It has been proposed that migratory leukocytes in the human endometrium have the ability to locally affect collagenase activity by secreting MMP-9 and MMP-2 directly, as reviewed by Fata *et al.* (2000). In the present study MMP-9 activity as demonstrated by *in situ* zymography was associated with eosinophil granulocytes. Eosinophils have been shown to regulate fibroblasts and stimulate collagen synthesis and it has been proposed that the MMP-9 they release accounts for tissue degradation during menstruation (Salamonsen & Woolley 1999). Neutrophils are known to secrete MMP-9 among other factors and play an important role in the cascade leading to endometrial tissue remodelling (Lathbury & Salamonsen 2000). In the present study active MMP-9 was not demonstrated to reside in neutrophils, even if it is highly likely that much of the MMP-9 detected in uterine lavages was secreted by neutrophils (Vincent *et al.* 1999). A similar phenomenon has been reported in bullous pemphigoid lesions, where both neutrophils and eosinophils demonstrated immunoreactive latent MMP-9 but only the latter exhibited a signal for gelatinase mRNA (Ståhle-Bäckdahl *et al.* 1994). As reported by Archer & Hirsch (1963) half of the total cell protein in the horse eosinophil is localised in the granules and its enzymes are contained or associated firmly with those granules, and indeed no gelatinase activity was demonstrated by the *in situ* method in the stroma outside of eosinophil granules. Once MMP has been released from granules it is activated, but once it has acted upon its substrates in the ECM it is likely to be rapidly inhibited by local TIMPs or degraded. This would explain why the secreted MMP is largely undetectable by *in situ* zymography.

The activity of the gelatinases MMP-2 and MMP-9 has been associated with asbestos-induced inflammation and subsequent fibrosis of the murine lung. Neutrophilic invasion of lung tissue was associated with the most prominent increase in MMP-9 activity whereas MMP-2 had a more prolonged expression pattern and its active form was specially associated with the chronic fibrosis phase (Tan *et al.* 2006). MMP-2 has been immunolocalised in areas of endometrial fibrosis (Walter *et al.* 2005) and it is possible that a prolonged induction of MMP-2 as a reaction to local inflammatory stimuli could contribute to the development of endometrial fibrosis. The present study centred on the normal self-limiting uterine inflammatory response in mares that did not have signs of endometrial fibrosis. Therefore an interesting step in investigating the pathogenesis of endometrial fibrosis would be to detect the activities of MMP-2 and MMP-9 in mares with active, ongoing fibrotic endometrial changes.

4.4 Conclusion

In conclusion, this study has demonstrated that the secretion of MMP-2 and MMP-9 into the uterine lumen is minimal during the normal equine oestrous cycle, although increased inhibitory activity is seen by TIMP-2 during dioestrus. The secretion of MMP-2 and MMP-9 into the uterine lumen is dramatically increased during endometritis and TIMP-2 activity is decreased at the same time. The activity of MMP-9 was demonstrated to reside in cytoplasmic granules of endometrial eosinophils, and gelatinase activity was not demonstrated in other cell types using *in situ* zymography. These results show that MMP secretion during endometritis is highly regulated and brought about by specialised inflammatory cells. An alteration

in the regulation of these MMPs may lead to the excess deposition of collagen resulting in endometrial fibrosis.

5 Chapter 5:

Endometrial fibrosis and correlating factors in Icelandic mares

5 Endometrial fibrosis and correlating factors in Icelandic mares

The histology of the endometrium of the Icelandic horse has not previously been reported. As an extensively managed, largely genetically isolated equine breed it provides a good model for the study of factors that affect reproductive efficiency without the additional complication of selective breeding and infectious causes of infertility. The breeding season of the Icelandic horse lasts from May until September and during the first month mares are bred at hand and at pasture, after which all mares are generally bred at pasture (Davies Morel & Gunnarsson 2000). Overall, the reproductive efficiency of Icelandic mares is good, and breeding occurs naturally with stallions being turned out with a herd of mares.

The Icelandic horse is the only equine breed found in Iceland. Traditionally, Icelandic horses are kept in a free-range manner in large herds and the majority of mares are bred by natural service at pasture. These horses are versatile, and are used for meat production, riding and as working horses. With increasing demand for good riding horses, organised breeding methods have been implemented and therefore the population might be seen as two subpopulations, kept in different farming systems (Hugason *et al.* 1985). Meat-producing mares often produce a foal every year until an advanced age with less of the fertility problems often seen in ageing thoroughbreds. However, in a retrospective study of the fertility of the overall population of Icelandic horses, mares younger than 6 years and older than 19 years were less fertile than mares 7-18 years old (Hugason *et al.* 1985). This upper age limit is relatively high compared to thoroughbreds that have been reported to show

signs of decreased fertility from the age of 14 (Morris & Allen 2002). A pregnancy rate of 82% and a live foal rate of 81% have been reported in pasture-bred Icelandic mares (Hugason *et al.* 1985). In a study carried out on cross-bred pasture bred mares, hormonal synchronization of oestrus yielded even higher conception and foaling rates, 88-97% and 72-94%, respectively (Bristol 1987). As most Icelandic mares are bred under extensive conditions, it is not common to carry out early pregnancy diagnosis and therefore it is possible that some conceptions were not discovered if they resulted in abortion before the mare was examined. Only one (0.02%) pregnancy of 4635 was reported to be a twin pregnancy, that is a very low figure compared to a reported 6.8% in the thoroughbred (Morris & Allen 2002). It is possible that in other cases, twinning was not discovered by the owners due to late pregnancy diagnosis and most mares foaling at pasture. The traditional management of Icelandic mares has involved conscious or unconscious selection for fertility and therefore it is interesting to investigate the prevalence of endometrial degeneration in this population.

A large proportion of the Icelandic horse population is registered in World*Fengur*, The Studbook of Origin for the Icelandic Horse that is designed and operated by the Farmers Association of Iceland and The International Federation of Icelandic Horse Associations. The inbreeding coefficient is included for many individuals in the database and is calculated by computing the relationship (R) between the selected parents by a recursive method described previously (Ter Heijden *et al.* 1977). The inbreeding coefficient (F) is half the coefficient of relationship (R). Both coefficients are multiplied by 100 and expressed as a percentage. The average inbreeding

coefficient is about 2.8% and matings resulting in inbreeding coefficients over 5% should be avoided, according to World*Fengur*.

As an overwhelming proportion of Icelandic mares are bred using traditional extensive methods, studies have not been done on the uterine health of Icelandic mares. Therefore, endometrial biopsies are hardly ever collected and it is not known if endometrial degeneration exists in this population. Furthermore, these mares are a valuable source of material in the effort to identify a possible heritability of this condition as selective breeding has been practiced for a relatively short period and to a limited extent, and inbreeding coefficients are known for a large proportion of mares. The study aimed to investigate if histological signs of endometrial degeneration could be identified in a population sample of the Icelandic horse. Also, in the case of endometrial fibrosis, if any of the previously suggested aetiological factors could be correlated with the condition in this population.

5.1 Materials and methods

5.1.1 Animals

Fourty-four Icelandic mares were used in this study, with samples collected from 26 mares in 2005 and 22 mares in 2006. Twenty-nine of the mares had a history of reproductive problems whereas 15 mares were reproductively normal. Mares classified as having reproductive problems were of two subcategories (**Table 5.1**). One subcategory included nonparous mares that had been unsuccessfully bred for at least two previous breeding seasons (15 mares). In the other subcategory were parous mares that had been bred with varying results, and had experienced two or

more consecutive unsuccessful breeding seasons in previous years (17 mares). The mares classified as without reproductive problems were either multiparous mares with a history of successful breeding (6 mares) or normal maidens (9 mares). The inbreeding coefficient was retrieved for 43 mares from World*Fengur* and ranged from 0 to 17.23%.

Table 5.1 Animals used in the study of Icelandic mares. Mares were defined as either with or without a history of reproductive problems. Mares with reproductive problems were further divided into nonparous mares in spite of repeated breeding attempts and parous mares with fertility problems in at least two preceding breeding seasons. Mares without a history of reproductive problems were divided into maidens that had never been bred and successful multiparous mares that had produced foals after every breeding attempt.

	Reproductive problems		**No reproductive problems**	
Fertility subcategory	**Nulliparous unsuccessful**	**Parous but recent fertility problems**	**Multiparous successful**	**Maiden**
Number of mares (percentage)	12 (27%)	17 (39%)	6 (14%)	9 (20%)
Average age	13 years	19 years	18 years	10 years
Age range	8-21 years	11-27 years	11-23 years	5-23 years
History of abortion	3	6	0	0
Clinical endometritis diagnosed during study	1	6	2	0

In the spring of 2005 and 2006, after the onset of cyclical activity, the mares were brought to Hólar University College in Hjaltadalur, Iceland and underwent a reproductive examination before samples were collected.

5.1.2 Clinical examination

Transrectal palpation and ultrasonography were carried out on all mares to monitor the oestrous cycle by observing ovarian follicle growth and uterine oedema. Most of the mares had been kept on pasture, and were to be introduced into a breeding herd with a stallion following the sample collection. Behavioural oestrus is

an easy indicator of commenced cyclic activity and so as not to reduce the number of oestrous cycles available for breeding, samples were collected at the first behavioural oestrus, as this would enable breeding at the next oestrus. External genitalia were inspected and internal genital organs (cervix, uterus and ovaries) were palpated transrectally, and findings were recorded. Subsequently, transrectal ultrasonography was carried out on the uterus and ovaries. The uterus was examined for oedema and intrauterine fluid as described in **2.1.2**. If clinical endometritis was diagnosed by identifying intrauterine fluid prior to sample collection, mares received treatment (see **2.2.4**). All samples were collected in oestrus, as determined by clinical signs as described in **2.1.1**. In the absence of these signs, mares were monitored daily by ultrasonography until they were confirmed to be in oestrus.

5.1.3 Sample collection

Before samples were collected the perineal area was prepared as described in **2.1.3**. Uterine lavage was performed by introducing isotonic saline through a uterine catheter via the relaxed cervix, as described in **2.1.5**. A sample (15ml) of the retrieved fluid was collected and prepared for storage until analysed (see **2.1.5**). Endometrial biopsies were collected (see **2.1.6**) and fixed in formol saline (see **2.1.6.2**). Two different staining methods were applied to endometrial biopsies; H&E (see **2.6.2**) and picrosirius red (see **2.6.3**). After all samples had been collected, all of the mares were returned to their owners and 36 of them were kept in different herds with a stallion for natural breeding at pasture.

5.1.4 Histopathology assessment

Each H&E stained endometrial biopsy was graded according to the subjective four-grade system as described by Kenney and Doig (1986). The grading of biopsies was carried out blindly by two clinicians. The area of each biopsy was then assessed by applying microscopic reticules at low power (10X objective) and estimating the length and breadth of the section. As well as assessing the Kenney category, a number of histological factors were assessed: The density of dilated glands and number of glands with periglandular fibrosis in the whole biopsy section were assessed by counting the total number of dilated and fibrotic glands and dividing by the area. The maximum gland diameter in each sample was estimated by applying reticules to the gland with the largest lumen in each biopsy. The presence or absence of endometrial fibrosis and acute endometritis as diagnosed using histology were recorded as binomial variables.

5.1.5 Detection of MMP-2, MMP-9 and TIMPs in uterine lavages

The activities of MMP-9 and MMP-2 in uterine lavages were demonstrated by gelatin substrate zymography (see **2.4.3**) and the activities of TIMPs were assessed by reverse zymography (see **2.4.4**). Due to the diluting effect of the lavage fluid, samples were concentrated up to achieve the correct dilution for the zymography protocol. This was achieved by freeze-drying (see **2.4.2**) and reconstituting each sample according to the amount of lavage fluid originally introduced into the uterine lumen.

5.1.6 Quantitative analysis of collagen fibres in endometrial biopsies

From each mare, one biopsy section stained with picrosirius was analysed for collagen content as described in **2.6.3**. The values obtained for each field were randomised to ensure objective analysis of the relationship between the area analysed and the optical density obtained.

The response variables used for statistical analysis were i) the optical density of collagen fibres and ii) the subjective category (Kenney category). Predictive variables were either clinical, histological or uterine lavage derived factors as displayed in **Table 5.2**.

Table 5.2 Predictive variables used in the statistical analysis factors correlated with endometrial fibrosis.

Clinical variables	Histological variables	Uterine lavage variables
Age	Kenney category	latent MMP-2 activity in uterine lavages
Parity	Optical density of collagen fibres	latent MMP-9 activity in uterine lavages
Reproductive problems Y/N	Histological endometritis Y/N	TIMP-2 activity in uterine lavages
Reproductive history	Number of dilated glands	
Inbreeding coefficient	Maximum glandular diameter	
Clinical endometritis	Endometrial fibrosis Y/N	
	Number of fibrotic glands	

5.1.7 Statistical analysis

In assessing the collagen content of endometrial sections by the picrosirius-polarization method, ten medium-power (16X objective) fields were assessed. The

area analyzed in each field varied due to the varying number and size of glandular areas that were subtracted from the analyzed area. In order to assess the variability in picrosirius value per area between the ten fields from each endometrial section, the coefficient of variation were calculated. In all but one mare this was relatively large at greater than 15%. In addition, linear regression of size of area analysed and optical density obtained revealed statistically significant positive linear relationships for four mares and a negative linear relationship for one mare, whereas no significant relationship was discovered for the other mares. Therefore, to proceed with the analysis the measured area was entered as a covariate to account for variations in optical density due to variations in measured area.

Proceeding with the statistical analysis, the total sum of the ten optical density values and corresponding measured areas were used as response variable and covariate, respectively. In order to evaluate the relationships of the numerical predictive variables (age, parity, inbreeding coefficient, Kenney category, fertility category, density of dilated glands, density of fibrotic glands, maximum diameter of glands, activity of MMP-2, activity of MMP-9 and activity of TIMP-2) to the optical density of collagen fibres, univariate linear regression analyses were carried out. For the categorical predictive variables (whether or not mares were classified as problem mares, whether or not there were clinical signs of endometritis, whether or not there were histological signs of endometritis, whether or not there was fibrosis on subjective analysis of H&E sections) univariate analyses of variance were carried out. Any factors with p values ≤ 0.1 were then put into a multivariate analysis of covariance to evaluate the relative importance of the predictive variables that were

statistically significant in the univariate analyses. In this final statistical model, statistical significance was taken as $p<0.05$. For any predictive variables that came up statistically significant in the multivariate analysis and had more than two categories, post-hoc multiple comparisons using Tukey contrasts were undertaken to determine where the differences between the groups lay.

A similar univariate and multivariate approach was adopted in the analyses of the Kenney category values and the other predictive variables above: however, as the Kenney category values for the mares only fell into two levels (IIA and IIB), general linear models with binomial errors were used instead. For one of the predictive variables (reproductive history) there were four levels, and to evaluate the suitability of an analysis with binomial errors for this predictive variable a Fisher's exact test was carried out before proceeding with a multivariate analysis of this variable.

5.2 Results

5.2.1 Histology

No mares had histological signs of severe chronic degenerative endometrial disease, and all mares were assigned to either category IIA or IIB (**Table 5.3**) on the basis of lymphocytic infiltrations or slight periglandular fibrosis. Therefore, no mares were assigned to the two extremes of the four-factor assessment system. The average age of mares in category IIA was 14.79 years (5-23 years) and the average age of category IIB mares was 16.28 years (7-27), see **Figure 5.1 a**. The average parity of category IIA mares was 3.32 foals (0-12) and the average parity of mares in category IIB was 6.56 foals (0-19), see **Figure 5.1 b**.

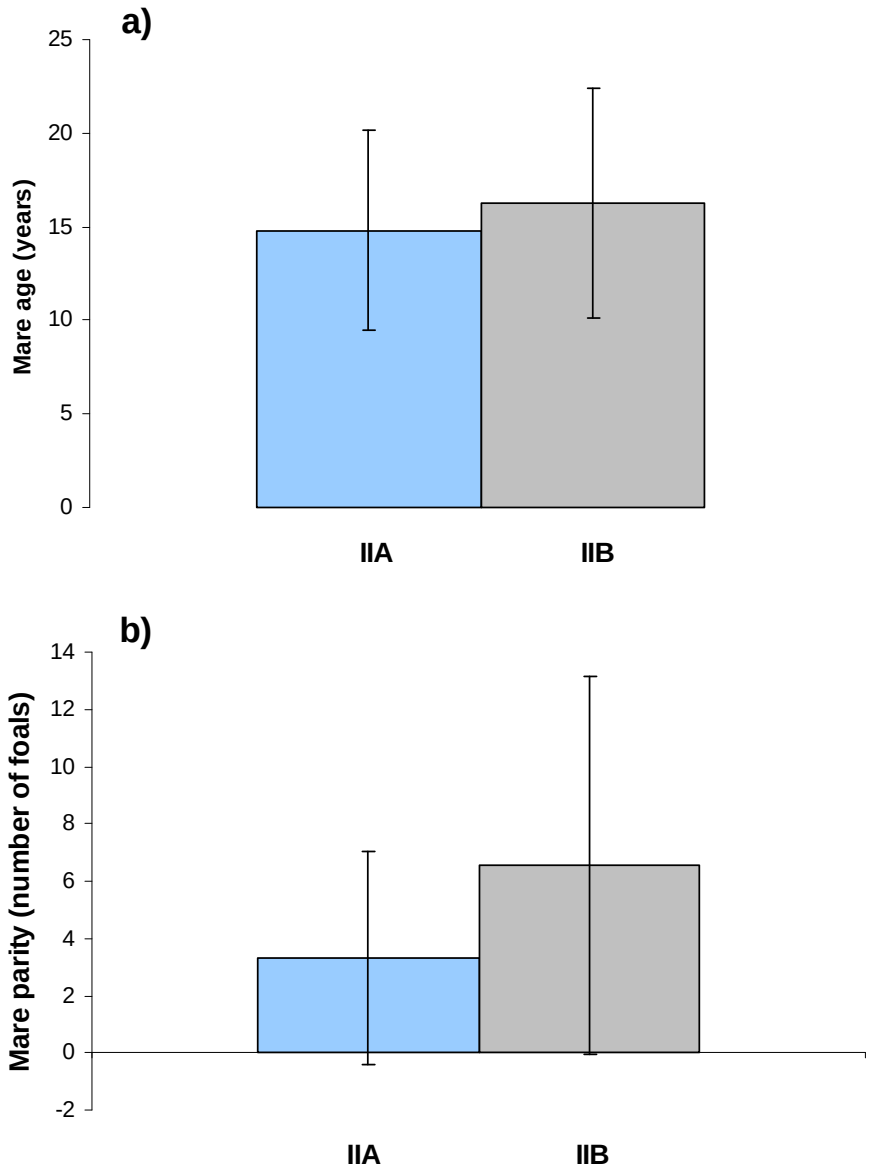


Figure 5.1 Average age and parity of mares included in the study. Average age (a) and average parity (b) of Icelandic mares classified in each of the Kenney categories IIA and IIB.

Table 5.3 Number of mares categorised into Kenney categories IIA and IIB and their distribution by fertility category.

	Maiden mares	Multiparous normal	Parous, recent fertility problems	Nonparous, unsuccessfully bred	Total
Kenney cat IIA	7	1	12	7	**27**
Kenney cat IIB	2	5	5	5	**17**
Total	**9**	**6**	**17**	**12**	**44**

5.2.2 Activity of MMP-2, MMP-9 and TIMP-2 in uterine lavages

Gelatin zymography demonstrated the activity of MMP-2 and MMP-9 in all uterine lavages. Reverse zymography demonstrated varying activity of TIMPs, although TIMP-2 was the only TIMP clearly detected in all but one mare and therefore the only TIMP analysed in this study.

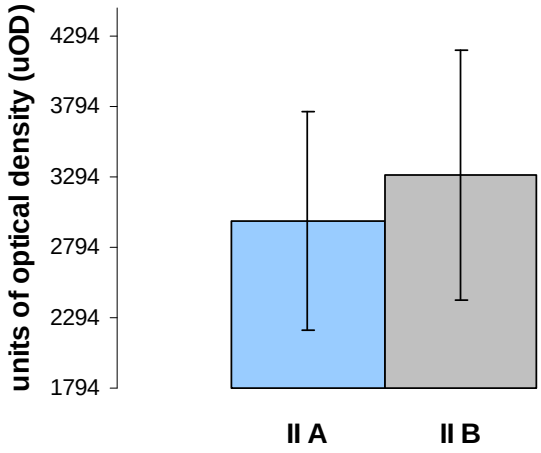


Figure 5.2 Optical density of collagen fibres by Kenney category. Optical density of collagen fibres indicates the amount of collagen deposition in equine endometrial sections. Optical density of collagen fibres tends to be higher in endometrial sections from mares in Kenney category IIB.

5.2.3 Optical density of collagen fibres in endometrial biopsies

The sum of optical density of collagen fibres in the ten fields analysed from each section was measured in units of optical density (**uOD**) and ranged from 1794 to 5028 uOD. The range of optical density of collagen in endometrial sections from mares in Kenney category IIA was 1794 to 4790 uOD, whereas Kenney category IIB mares ranged from 2058 to 5028 uOD (**Figure 5.2**).

5.2.4 Analysis of univariate and multivariate analyses

The univariate analysis on the optical density of collagen fibres (**Table 5.4**) demonstrated a statistically significant negative relationship between optical density of collagen fibres and the presence of reproductive problems (p=0.008), a statistically significant positive relationship between optical density and inbreeding coefficient (p=0.02) and a statistically significant relationship between optical density of collagen fibres and fertility category (p=0.017). When 'reproductive problems Y/N' and inbreeding coefficient were entered into a multivariate model (**Table 5.5**), the statistical significance was entirely explained by inbreeding coefficient (p=0.031 when entered first and p=0.018 when entered second) and no statistical significance was accredited to reproductive problems (p=0.063 when entered first and p=0.121 when entered second). Post-hoc Tukey contrasts demonstrated that optical density of collagen fibres in the endometrium of maiden mares was statistically significantly (p=0.01) higher than that of parous mares with recent reproductive problems (**Figure 5.3**), but when inbreeding coefficient was entered as the first predictive variable, this significance was lost (p=0.316) due to the overriding importance of inbreeding coefficient in this significance (**Figure 5.4**).

Table 5.4 Results of univariate analyses on two response variables and various predictive variables. Significance was taken when p<0.05 (values in bold), and predictive variables that produced p values of ≤0.1 were entered into a further multivariate analysis. Levels of statistical significance; p<0.05: +, p<0.01: ++, p<0.001: +++

	Response variable			
	Optical density of collagen		Kenney category	
Predictive variable	p-value	Level of stat sign	p-value	Level of stat sign
Age	0.429	NS	0.268	NS
Parity	0.186	NS	**0.030**	**+**
Kenney category	0.241	NS		
Reproductive history	**0.017**	**+**	0.076	NS
Reproductive problems Y/N	**0.008**	**++**	0.433	NS
Inbreeding coefficient	**0.020**	**+**	0.111	NS
Clinical endometritis	0.446	NS	0.250	NS
Histological endometritis	0.668	NS	**0.032**	**+**
Histological endometrial fibrosis	0.375	NS	**0.009**	**++**
Number of dilated glands	0.602	NS	0.269	NS
Maximum glandular diameter	0.186	NS	0.660	NS
Number of fibrotic glands	0.933	NS	0.132	NS
latent MMP-9 activity in uterine lavages	0.332	NS	0.263	NS
latent MMP-2 activity in uterine lavages	0.982	NS	0.772	NS
TIMP-2 activity in uterine lavages	0.818	NS	0.408	NS

Table 5.5 Multivariate analyses on optical density of collagen fibres. With both methods it was demonstrated that the statistical significance attributed to inbreeding coefficient explains any statistical significance attributed to reproductive problems Y/N or reproductive history. Significance was taken when p<0.05 (values in bold). Levels of statistical significance; p<0.05: +, p<0.01: ++, p<0.001: +++

Analysis of covariance

Predictive variable	Inbreeding coefficient		Reproductive problems Y/N		Fertility category	
	p-value	Level of stat sign	p-value	Level of stat sign	p-value	Level of stat sign
Inbreeding coefficient +Reproductive problems Y/N	**0.018**	**+**	0.121	NS		
Reproductive problems Y/N +Inbreeding coefficient	**0.031**	**+**	0.063	NS		
Inbreeding coefficient +Reproductive history	**0.017**	**+**			0.168	NS
Reproductive history +Inbreeding coefficient	**0.036**	**+**			0.095	NS

Post-hoc Tukey contrasts on Reproductive history	estimate	p-value	Level of stat sign
Multiparous-maiden mares	-437	0.663	NS
Recent problems-maiden mares	-1031	**0.010**	**++**
Unsuccessfully bred-maiden mares	-628	0.237	NS
recent problems-multiparous	-595	0.319	NS
Unsuccessfully bred-multiparous	-192	0.950	NS
Unsuccessfully bred-recent problems	403	0.481	NS

Post-hoc Tukey contrasts on Inbreeding coefficient+Reproductive history	estimate	p-value	Level of stat sign
Multiparous-maiden mares	-55	0.999	NS
Recent problems-maiden mares	-605	0.316	NS
Unsuccessfully bred-maiden mares	-137	0.982	NS
recent problems-multiparous	-550	0.335	NS
Unsuccessfully bred-multiparous	-81	0.995	NS
Unsuccessfully bred-recent problems	468	0.353	NS

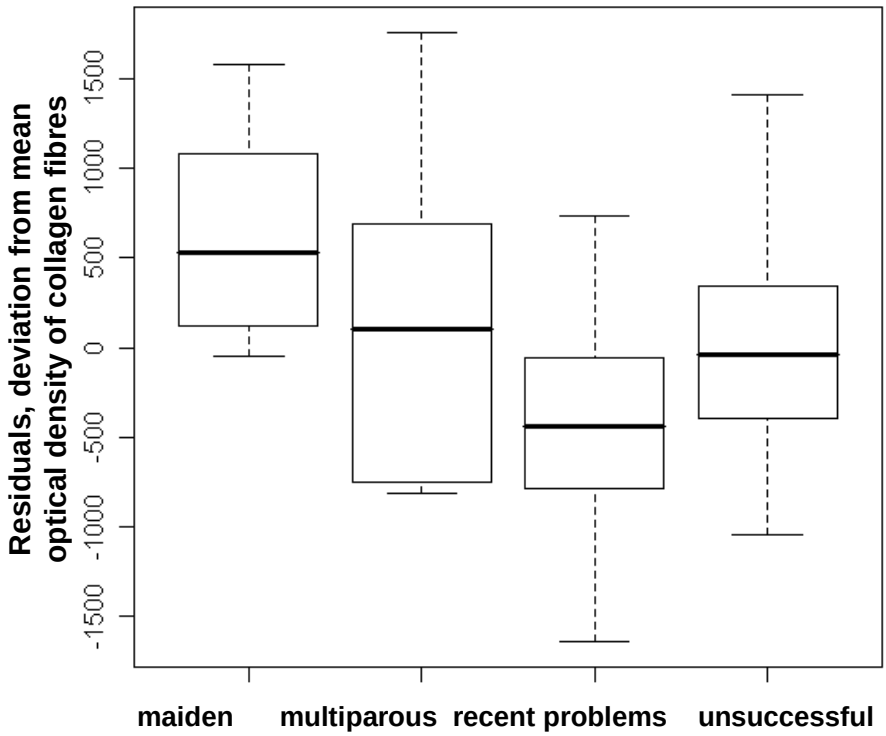


Figure 5.3 Boxplot illustrating of residuals for the four fertility categories. The four fertility categories (maiden mares, multiparous mares without fertility problems, parous mares with recent fertility problems, and nonparous mares unsuccessfully bred) were entered into multivariate analysis. The optical density of collagen fibres was statistically significantly (p=0.01) higher in the endometrium of maiden mares than in parous mares with recent fertility problems.

Statistical significance was not found (p=0.24) between Kenney category and optical density of collagen fibres. The univariate analysis of Kenney category (**Table 5.4**) demonstrated a statistically significant positive relationship with parity (p=0.03), histological endometritis (p=0.032) and histological fibrosis (p<0.009). Relationships were demonstrated with inbreeding coefficient (p=0.111) and fertility category (p=0.076), and although not statistically significant these factors were also analysed further.

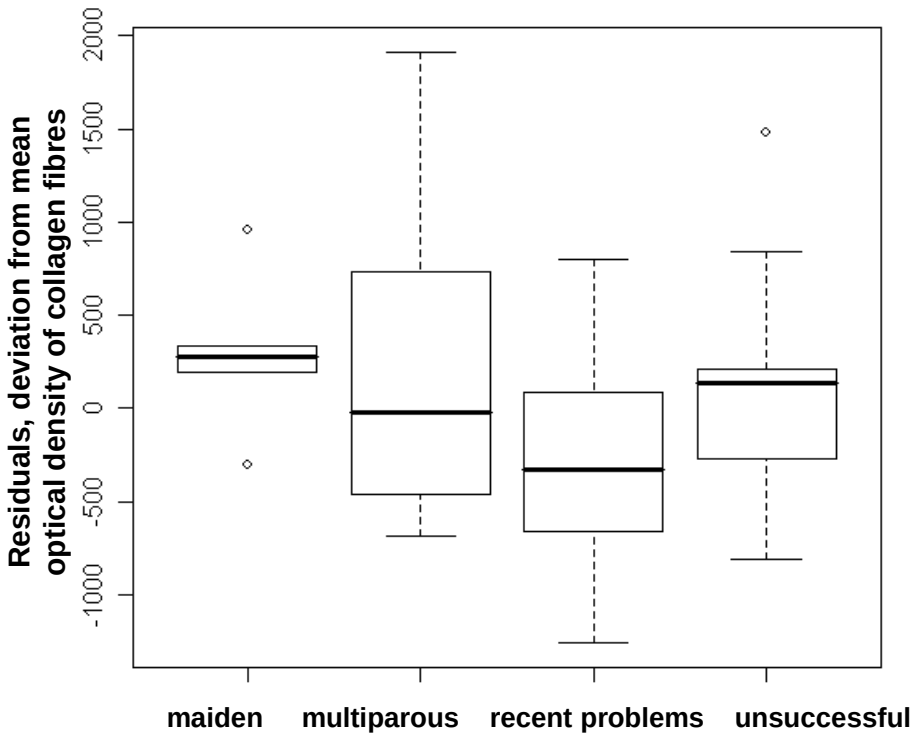


Figure 5.4 Boxplot of residuals for the four fertility categories after taking inbreeding coefficient into account. The four fertility categories (maiden mares, multiparous mares without fertility problems, parous mares with recent fertility problems, and nonparous mares unsuccessfully bred) were then entered into multivariate analysis along with inbreeding coefficient that removed the statistical significance previously demonstrated between maiden mares and parous mares with recent fertility problems.

The multivariate analysis with binomial errors demonstrated that there was a statistically significantly positive relationship between Kenney category and the subjective diagnosis of endometrial fibrosis (p=0.017) and histological endometritis (p=0.003). The relationship between Kenney category and mare parity was not statistically significant (p=0.062) once significance had been explained by subjective endometrial fibrosis and histological endometritis. The same result was found when inbreeding coefficient was entered in the multivariate analysis (p=0.407) with endometrial fibrosis and histological endometritis. In the multivariate analysis, fertility category did not yield reliable results, and when this predictive variable was analysed by Chi-square and Fisher's exact tests it was evident that this four-category variable was not suitable for binomial analysis due to limited numbers in at least one of the categories.

5.3 Discussion

This study has for the first time reported on the endometrial histology of Icelandic mares. In spite of the wide array of ages, parities and fertility categories of mares chosen for this study, all fell into only two of the middle Kenney categories. The oldest mare in the study was 27 years old, had produced 19 live foals and was assigned Kenney category IIB, and six mares aged 19-23 fell into category IIA. These mares are well beyond the minimum acceptable age for Kenney category III that according to Ricketts & Alonso (1991) is 17 years. The age range of mares in category IIA was 5-23 years and included five mares under the minimum age of 9 years for which category IIA is acceptable according to Ricketts & Alonso (1991). From a practical standpoint, Kenney category I is rarely assigned due to the

prevalence of slight lymphocytic infiltrations in the endometrium of most reproductively normal mares. Indeed, the presence of lymphocytic infiltrations in the stratum compactum of endometrial sections were shown in **chapter 3** to increase during oestrus in normal mares and therefore their presence does not necessarily represent permanent fertility problems for the mare. Therefore, it may be the case that some mares assigned to Kenney category IIA exhibit transient lymphocytic infiltrations due to an influence of the oestrous cycle and so should rather be classified in Kenney category I. Nevertheless, it is recommended to obtain endometrial biopsies for categorization by the Kenney scale during dioestrus (Ricketts 1975b) and this is incidentally the stage where less lymphocytic infiltrations can be expected. The influence of this cyclical difference on the Kenney categorization may therefore not be clinically relevant. In a study utilising picrosirius for the morphometric analysis of equine endometrial biopsies it was recommended to obtain endometrial biopsies during oestrus, as during this stage diffuse endometrial oedema and minimal nonfibrotic huddling of glands displays a clear contrast between areas with an abundance or scarcity of collagen fibres (Evans *et al.* 1998).

In their study Evans *et al.* (1998) found a statistically significant correlation between volume fraction of collagen and the Kenney category system. This could not be demonstrated in the present study, as Kenney category did not correlate with the optical density of collagen fibres. This may be due to the fact that the population sample was small and mares did not exhibit enough variation in Kenney categorization. However, the diagnosis of endometrial fibrosis by subjective analysis

of H&E stained sections by light microscopy failed to correlate with the optical density of collagen fibres in picrosirius stained sections. This may be due to the erroneous interpretation of fibroplasia (described as layers of fibroblasts rather than collagen (Walter *et al.* 2001) as fibrosis. The method used to detect collagen fibres using picrosirius is considered to be specific and accurate and a similar computer-aided analysis of digital grey-scale images using light microscope was demonstrated to be effective in diagnosing collagen fibres in rat tongue (Amenábar *et al.* 2006).

Due to the fact that no mares were assigned to the two extreme categories of the Kenney system the statistical analysis could be expected to be problematic as the standard deviation of optical density might prove to be too large within each category to show differences between the two. As already mentioned a statistically significant difference was demonstrated in the collagen fraction between all four categories, but interestingly the increase in collagen percentage was considerably larger between categories IIB and III than between categories IIA and IIB (Evans *et al.* 1998). In the present study the range of collagen amount as expressed by optical density overlapped between the two categories and a correlation with Kenney category was not made. It was demonstrated that even in old mares, multiparous or maiden, degenerative changes to the extent of Kenney category III may not develop in the Icelandic mare. It would be interesting to conduct a similar study on thoroughbred or other light breed mares using this method of picrosirius-polarization analysis to determine the optical density of collagen fibres in mares classified as category III. The application of the Kenney scale to endometrial biopsies from Icelandic mares is questionable. The scale has been shown to be applicable and clinically relevant in

mares of other breeds, especially thoroughbred and other light breeds, but even though it has predictive value for mare fertility it is recommended that other aetiological and clinical factors be taken into account when predicting mare fertility (Ricketts 1975b; Williamson *et al.* 1989; Troedsson *et al.* 1993a). For the assessment of collagen amount in the endometrium the picrosirius-polarization method is specific and precise and can be recommended over the subjective assessment of fibrosis in H&E stained endometrial sections for research purposes.

The positive correlation with Kenney category and the observation of endometrial fibrosis and signs of endometritis in H&E stained sections is not surprising as those are both factors by which a categorization by the Kenney scale is made. No correlation was found between age and Kenney category, and the correlation shown between Kenney category and parity in the univariate analysis was demonstrated to be insignificant when histological endometritis and subjective diagnosis of endometrial fibrosis had been taken into account. Numerous studies have previously demonstrated a positive correlation between age and Kenney category, whereas evidence tends to be conflicting when associating parity with Kenney category (Doig *et al.* 1981; Ricketts & Alonso 1991; Bracher 1997). Icelandic mares evidently develop less severe endometrial degenerative changes, and these changes occur at a higher age than corresponding changes in the thoroughbred. Therefore, it is unsurprising that a correlation was not found between the age or parity of these mares and the Kenney category that they were assigned.

No correlation was demonstrated between the amount of collagen or Kenney category and the activity of MMP-2, MMP-9 and TIMP-2 in uterine lavage fluid. This may be explained by the limited degree of endometrial degeneration in this study population that may fail to demonstrate subtle changes in the activity of MMPs and TIMPs that might occur in more extreme endometrial fibrosis. It is also likely that endometrial fibrosis is not continuously taking place, and therefore the enzymes taking part in this process are not found in constantly increased amounts in the uterus. This relationship must be investigated in a breed known to develop endometrial fibrosis, and possibly by inducing endometritis in mares with the condition.

Univariate statistical analysis demonstrated that endometrial sections from mares defined as reproductively normal had larger amounts of collagen than mares defined as having reproductive problems. It would not have been surprising to discover an increase in collagen amount in mares with reproductive problem, so this result was unexpected. The group of reproductively normal mares was considerably smaller than the group of mares with reproductive problems and this could in some way explain this result. Additionally, reproductive normal mares included maiden mares, some of whom were middle-aged or old and might be expected to have increased amounts of endometrial collagen due to the lacking of the proposed protective effect of pregnancy against endometrial degeneration (Chevalier-Clément 1989). However, these older maiden mares did not have the highest amount of collagen, although it can be recommended to include a higher number of mares and possibly to divide maiden mares into age groups. However, this statistical significance disappeared

when this variable was entered into a multivariate analysis along with the inbreeding coefficient that was shown to be positively correlated with collagen amount in the univariate analysis. Therefore, inbreeding coefficient was found to be an overwhelmingly important influence on the deposition of collagen in the endometrium of Icelandic mares. The influence of inbreeding on the fertility of the thoroughbred was discussed by Morris and Allen (2002) and they proposed that the ability to predict the genetic compatibility of individual stallions and mares would improve the fertility of the thoroughbred. Increasing inbreeding coefficient has been correlated with reduced semen quality (sperm morphology and viability) in Shetland pony stallions, with the effect becoming evident at inbreeding coefficients of 2-5% and becoming increasingly obvious at higher levels of inbreeding (van Eldik *et al.* 2006). In another study a positive linear relationship was demonstrated between the incidence of retained placenta in Friesian mares and the inbreeding coefficient of the foal, but not with the inbreeding coefficient of the mare (Sevinga *et al.* 2004). The Friesian horse has a high prevalence of retained placenta and as the inbreeding coefficient of the foal was correlated with this condition, it was speculated that the pathogenesis might involve immunological factors between the foetus and dam (Sevinga *et al.* 2004). The pathogenesis of retained placenta in the cow involves changes in the regulation of MMP-2 in foetal and maternal placenta, and it is plausible that similar mechanisms take place to cause retained placenta in the mare. It is likely that endometrial fibrosis and retained placenta are caused by defects in the endometrial remodelling process, possibly involving the MMPs.

5.4 Conclusion

Icelandic mares do not develop endometrial degenerative changes to the same extent as mares of light breeds, such as thoroughbreds. The Kenney scale widely used to classify endometrial inflammatory and degenerative changes in other breeds does not appear to be useful in the clinical diagnosis of fertility problems in the Icelandic mare due to the low grade of endometrial degeneration demonstrated in this study population in spite of a wide range of age, parity and fertility status of the mares studied. Furthermore, the results of this study indicate that endometrial degeneration is not an important influence on the fertility of Icelandic mares, as no correlation was demonstrated between fertility problems and Kenney category or collagen amounts in the endometrium in the studied population. In spite of limited degenerative endometrial changes demonstrated in this population, it was shown that the amount of collagen in endometrial sections was positively correlated with the level of inbreeding. It is possible that inbreeding has detrimental effects on endometrial remodelling events, with collagen deposition occurring as a result.

6 Chapter 6:

Activities of MMP-2, MMP-9 and TIMPs in equine foetal fluids during the latter half of gestation and at parturition

6 Activities of MMP-2, MMP-9 and TIMPs in equine foetal fluids during the latter half of gestation and at parturition

In the last days before parturition, the equine placental microvilli are still growing longer and more branching is seen at the villous tips (Macdonald *et al.* 2000b). Lung maturation, including surfactant production occurs very late in gestation, and numbers of terminal bronchiolar ducts even increase postnatally in thoroughbreds (Pattle *et al.* 1975; Beech *et al.* 2001). Foals that are born without a minimum level of lung maturity may be at a higher risk of neonatal respiratory distress syndrome that calls for intensive treatment that has variable results (Dunkel *et al.* 2005). Communication exists between the amniotic compartment and lung lumen, and therefore the lungs contain amniotic fluid during gestation (Devlieger *et al.* 2002). The development of foetal tissues and placenta during this late surge of growth requires tissue remodelling although little is known about this process in the horse. In the pre-parturient period, the foetal membranes are likely to undergo some remodelling resulting in the weakening of their tensile strength to allow for rupture during parturition. It is possible that "red-bag" delivery (see **1.1.3.6**) is a result of the interruption of tissue remodelling of the foetal membranes. MMPs are expressed in the equine (Vagnoni *et al.* 1995), bovine (Maj & Kankofer 1997), ovine (Vagnoni *et al.* 1998) and human (Goldman *et al.* 2003) placenta and foetal membranes, as well as foetal fluids. Increased secretion of matrix metalloproteinases is involved in foetal membrane breakdown at parturition in several species, including humans and rats (Athayde *et al.* 1998). The activity of MMP-9 in human and rodent foetal membranes and amniotic fluids increases markedly with the onset of labour,

associating this enzyme with the rupture of foetal membranes and detachment from the endometrium (McLaren *et al.* 2000b; Locksmith *et al.* 2001).

The activity of MMP-9 and MMP-2 in late equine gestation and parturition has not previously been investigated. We hypothesise that tissue remodelling by MMPs takes place during the latter half of equine gestation and at equine parturition. Therefore, the aim of this study is to determine activities of MMP-9 and -2 and their inhibitors (TIMPs) in equine amniotic fluid throughout mid- to late pregnancy and during foaling.

6.1 Materials and methods

6.1.1 Study design and collection of amniotic fluid samples

A longitudinal study was used to determine the activities of MMP-2 and MMP-9 and TIMPs in equine amniotic fluid through mid- and late gestation, and foaling. Five groups of mares were used (**Table 6.1**). Group 1 (n=5) consisted of pony mares that were sampled in mid-gestation (days 191-236 of gestation). Group 2 (n=7) consisted of pony mares that were sampled late in gestation (days 294-336 of gestation), and whose pregnancy resulted in normal foaling at term (days 313-338 of gestation). Group 3 (n=7) consisted of pony mares that were sampled in late gestation (days 183-306 of gestation), but whose pregnancy resulted in abortion (<day 300 of gestation, n=5) or stillbirth (≥day 300 of gestation, n=2). Group 4 (n=10) consisted of pony mares sampled during spontaneous foaling at term. Group 5 (n=9) consisted of thoroughbred mares sampled during spontaneous foaling at term.

Amniotic fluid for groups 1, 2 and 3 was collected repeatedly (2 to 13 samples from each mare) from 19 pony mares at day 191-336 of gestation (0-145 days before birth) through indwelling catheters inserted under general anaesthesia as described in **2.3.1**. Allantoic and amniotic fluid samples were collected at foaling from another ten pony mares (group 4) and nine thoroughbred mares (group 5) at the spontaneous rupture of foetal membranes at normal term as described in **2.3.2**. All samples were prepared as described in **2.3.3**.

Table 6.1 Definition of the five groups of mares from which foetal fluid samples were collected. Sequential samples of amniotic fluids were collected from chronically catheterised pony mares (days of gestation), and allantoic and amniotic fluid samples were collected from foaling pony and thoroughbred mares

Chronically catheterised pregnant pony mares			**During foaling**	
			Pony mares	**Thoroughbred mares**
Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**
Normal mid gestation (day 191-236)	Normal late gestation (day 294-336)	Stillbirths + abortions (day 183-306)	Spontaneous foaling at term	Spontaneous foaling at term
5 mares, 36 samples total	7 mares, 35 samples total	7 mares, 27 samples total	10 mares, 10 samples total	9 mares, 9 samples total

6.1.2 Detection of MMP-2, MMP-9 and TIMP activity in foetal fluids

Activity of the gelatinases MMP-2 and MMP-9 in amniotic and allantoic fluid samples was detected using gelatin zymography as described in **2.4.3**. TIMP activity in amniotic and allantoic fluid samples was detected by reverse zymography

as described in **2.4.4**. Quantification of MMP and TIMP activity was carried out by densitometric analysis as described in **2.4.5**.

6.1.3 Statistical analysis of data

The activities of MMP-2, MMP-9 and TIMP-2 in foetal fluids were normally distributed and therefore parametric tests were used. In order to analyse the temporal change in the activities of MMP-2, MMP-9 and TIMP-2 in chronically catheterised mares in the period approaching foaling, generalised linear mixed effect models were used (Pinheiro & Bates 2000). In order to account for the repeated sampling from each mare in the period leading up to foaling, the gestational age was entered as a fixed effect and mare IDs were entered into the model as a random effect.

The last amniotic samples taken before parturition (1-3 days prior to parturition) from each of the chronically catheterised mares were then defined as pre-parturient samples. Pre-parturient activities of latent MMP-9, and active and latent MMP-2 were compared to activities of these same enzymes in actively foaling pony mares using generalised linear mixed effect models. Using this model it was possible to enter pre-parturient MMP activities prior to normal births on one hand and prior to stillbirths or abortions on the other. The activity of TIMP-2 was measured only in pre-parturient samples as well as samples taken from actively foaling mares. Therefore, these results were compared using unequal sample size two-sample *t*-tests due to the lack of repeated sampling. Finally, using generalised linear mixed effect models, the activities of MMP-2, MMP-9 and TIMP-2 in amniotic and allantoic fluids during foaling in pony and thoroughbred mares were compared as these were

repeated measures from the same animals. The statistical software packages used were Minitab Statistical Software, Release 14.1 (Minitab Inc., Philadelphia) and R 2.0.1 (Free Software Foundation Inc., Boston). Statistical significance was taken when $p<0.05$.

6.2 Results

All zymograms (**Figure 6.1 a**) showed bands corresponding to active and latent MMP-2 (66 and 72 kDa, respectively) and latent MMP-9 (92 kDa). A few samples demonstrated bands corresponding to active MMP-9, but this form of the enzyme seemed to be detected transiently due to its instability. Hence it was highly variable and unrepresentative of MMP-9 in its latent form so was not analysed quantitatively. As no statistically significant difference ($p=0.379$) was found in amniotic MMP-2 and MMP-9 activity between pregnancies resulting in abortion and those resulting in stillbirth, these were considered as one group for easier statistical analysis.

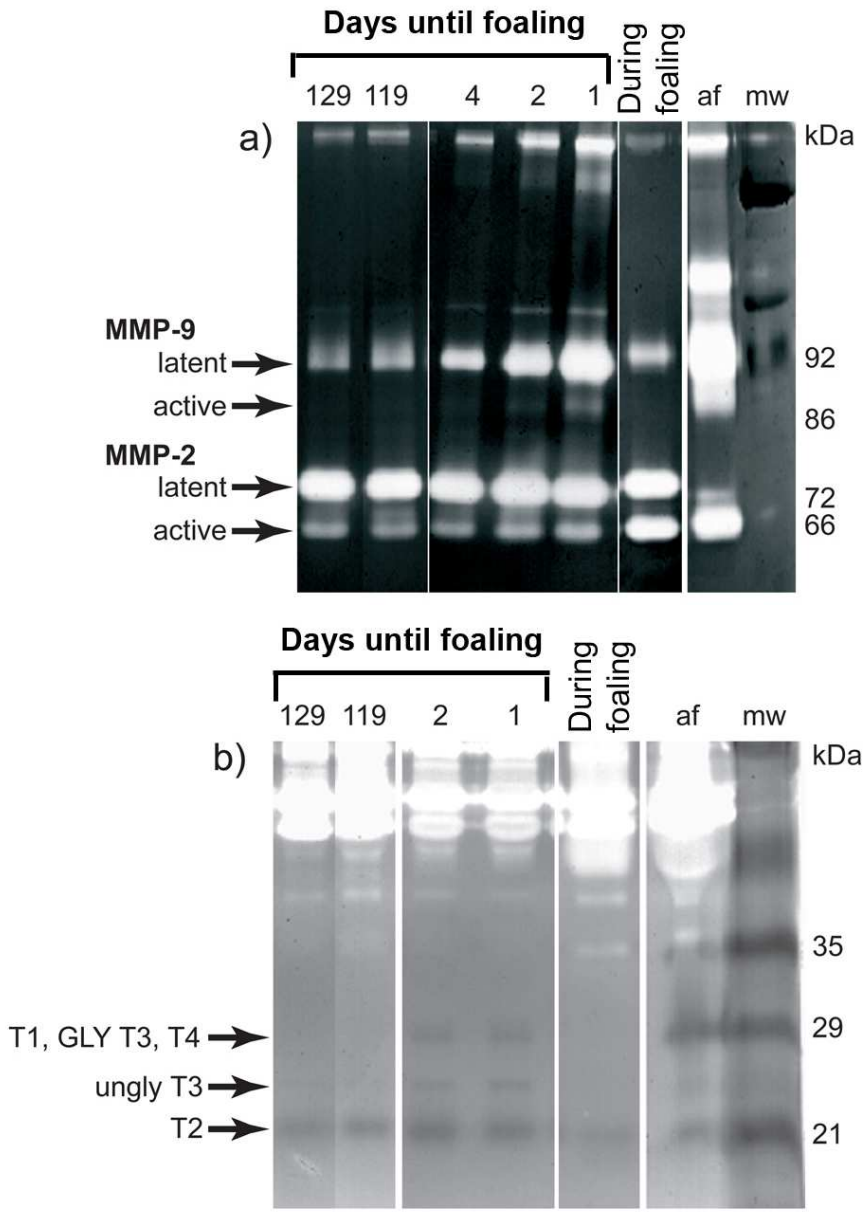


Figure 6.1 Zymogram of the activity of MMPs and TIMPs in equine amniotic fluid. a) Representative gelatin zymogram showing the activity of MMP-2 and MMP-9. b) Representative reverse zymography showing the activity of TIMPs with molecular weight 21 kDa (TIMP-2), 24 kDa (unglycosylated TIMP-3) and 29 kDa (TIMP-1, glycosylated TIMP-3 and TIMP-4); af: human amniotic fluid as a characterised control sample, mw: molecular weight markers.

Reverse zymography showed bands with molecular weights of 27-30, 24 and 21 kDa (**Figure 6.1 b**). The 21 kDa band representing TIMP-2 was the most intensively expressed and was found in all amniotic fluid samples. There was a lesser abundance in the 27-30 kDa band that consists of TIMP-1, glycosylated TIMP-3 and TIMP-4 that all have similar molecular weights and cannot be differentiated by reverse zymography, and in the 24 kDa band that contains unglycosylated TIMP-3.

6.2.1 MMP-9 activity in amniotic fluid during gestation and foaling

In mid-gestation the activity of latent MMP-9 fluctuated between sampling points (**Figure 6.2 a**) but there was no statistically significant gestational trend over the sampling period. There was a statistically significant increase in activity of latent MMP-9 ($p=0.002$) in late gestation when approaching normal foaling, but there was no statistically significant change ($p=0.781$) when approaching abortion or stillbirth. Approaching parturition, all analyses were performed based on the number of days prior to parturition rather than day of gestation. Approaching normal foaling (**Figure 6.2 b**), latent MMP-9 was present in increasing amounts ($p<0.001$), whereas there was no statistically significant change ($p=0.963$) in the activity of latent MMP-9 approaching stillbirth and abortion (**Figure 6.2 c**).

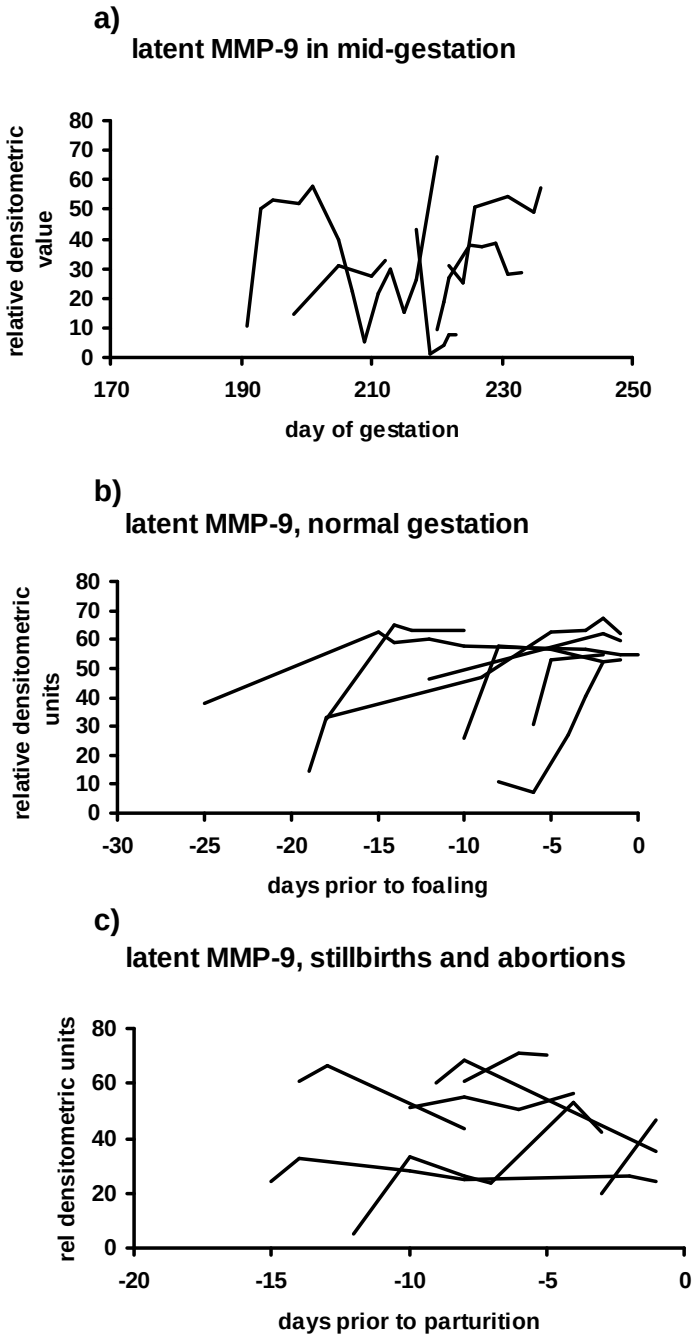


Figure 6.2 Activity of latent MMP-9 in equine amniotic fluid as demonstrated by gelatin zymography. a) Sequential samples of amniotic fluids throughout mid-gestation (Days 190 to 234 of gestation). b) Sequential samples taken from normal pre-parturient mares (25 to 1 day before foaling). c) Sequential samples taken from pre-parturient mares before (15 to 1 day before) stillbirth or abortion.

When MMP activities in pony mares during the normal pre-parturient period were compared with activities during foaling, the activity of latent MMP-9 was statistically significantly ($p<0.001$) higher than during foaling (**Figure 6.3 a**). Latent MMP-9 activity in the last 3 days before stillbirth or abortion was statistically significantly ($p<0.001$) higher than during foaling (**Figure 6.3 b**). When groups 2 and 3 were compared, the activity of latent MMP-9 in the pre-parturient period was statistically significantly ($p=0.002$) lower prior to stillbirth or abortion than before normal birth. The activity of latent MMP-9 was detected in amniotic fluid from thoroughbred and pony mares during foaling. The activity of latent MMP-9 was statistically significantly ($p=0.02$) higher in thoroughbred mares than in pony mares (**Figure 6.4 a**).

6.2.2 MMP-2 activity in amniotic fluid during gestation and foaling

As for latent MMP-9, there was a fluctuating pattern to the activity of latent MMP-2 through mid-gestation but there was no statistically significant gestational trend ($p=0.305$) over the sampling period. There was no statistically significant change in the activity of latent MMP-2 in late gestation when approaching normal parturition ($p=0.311$), but there was a statistically significant ($p=0.04$) reduction in latent MMP-2 activity when approaching abortion or stillbirth.

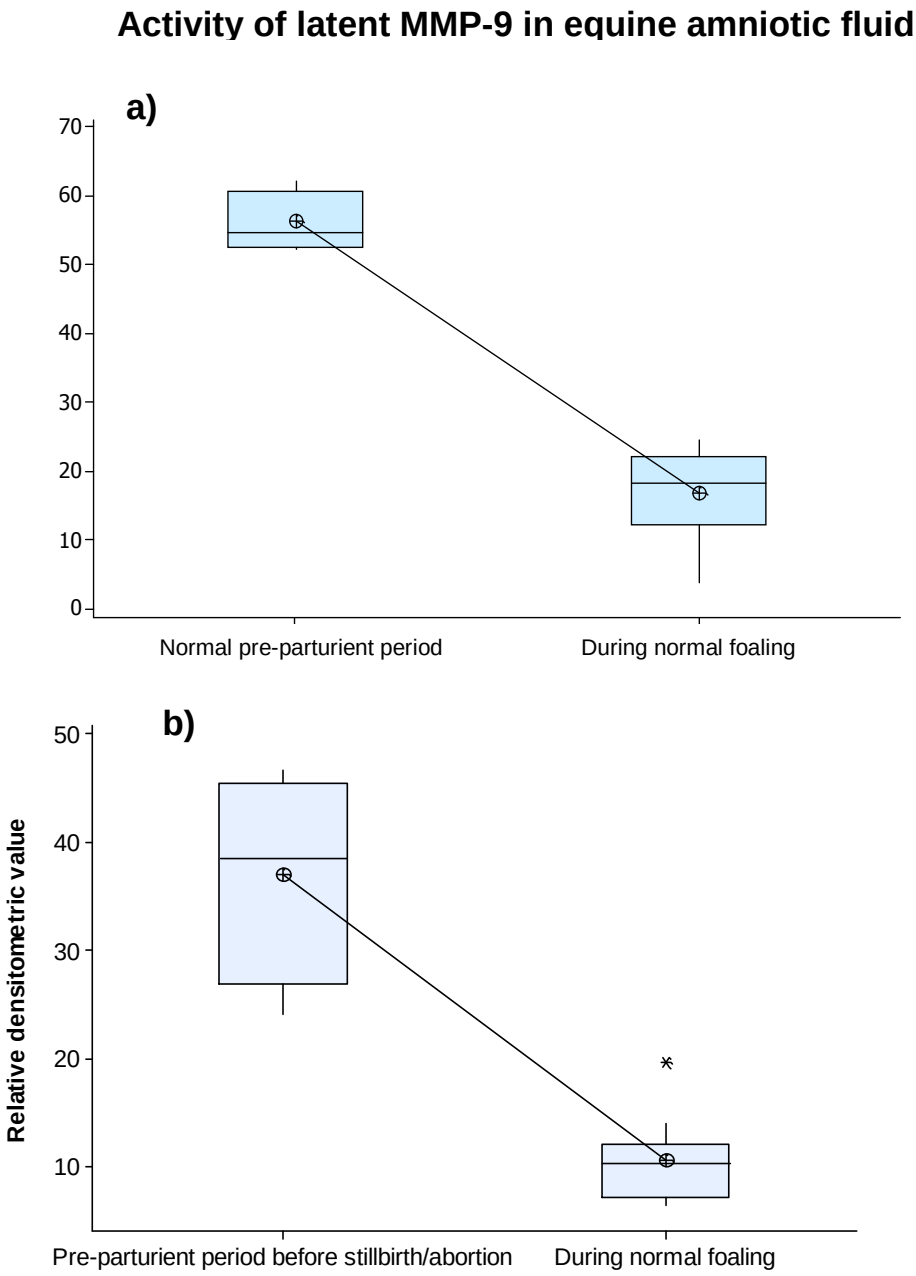


Figure 6.3 Activity of latent MMP-9 in equine amniotic fluid before and during foaling. a) Latent MMP-9 activity during normal foaling was statistically significantly ($p<0.001$) lower than during the pre-parturient period (1 to 3 days prior to parturition) preceding normal foaling. b) Latent MMP-9 activity during normal foaling was statistically significantly ($p<0.001$) lower than during the pre-parturient period preceding stillbirth or abortion.

Approaching normal foaling, latent and active forms of MMP-2 were detected in varying amounts in all samples, but there was no statistically significant ($p=0.433$) change in activities through the sampling period. This was true also when approaching stillbirth and abortion, and there was no difference ($p=0.15$) in activity when comparing these results to normal pre-parturient latent MMP-2 activity. The activity of latent MMP-2 during foaling in pony mares was not statistically different ($p=0.25$) from the activity in the normal pre-parturient period, but was statistically significantly higher than when approaching stillbirth or abortion ($p=0.004$). The activity of active MMP-2 was statistically significantly lower in the pre-parturient periods before stillbirth or abortion ($p<0.001$) and before normal foaling ($p=0.03$) than during foaling in pony mares. There was no statistically significant difference ($p=0.34$) in active MMP-2 activity in the pre-parturient periods before stillbirth or abortion and normal foaling.

MMP-2 was detected in amniotic fluid from foaling thoroughbred and pony mares, with statistically significant breed differences. The active and latent (**Figure 6.4 b**) forms were statistically significantly ($p=0.018$ and $p<0.001$, respectively) lower in thoroughbred mares.

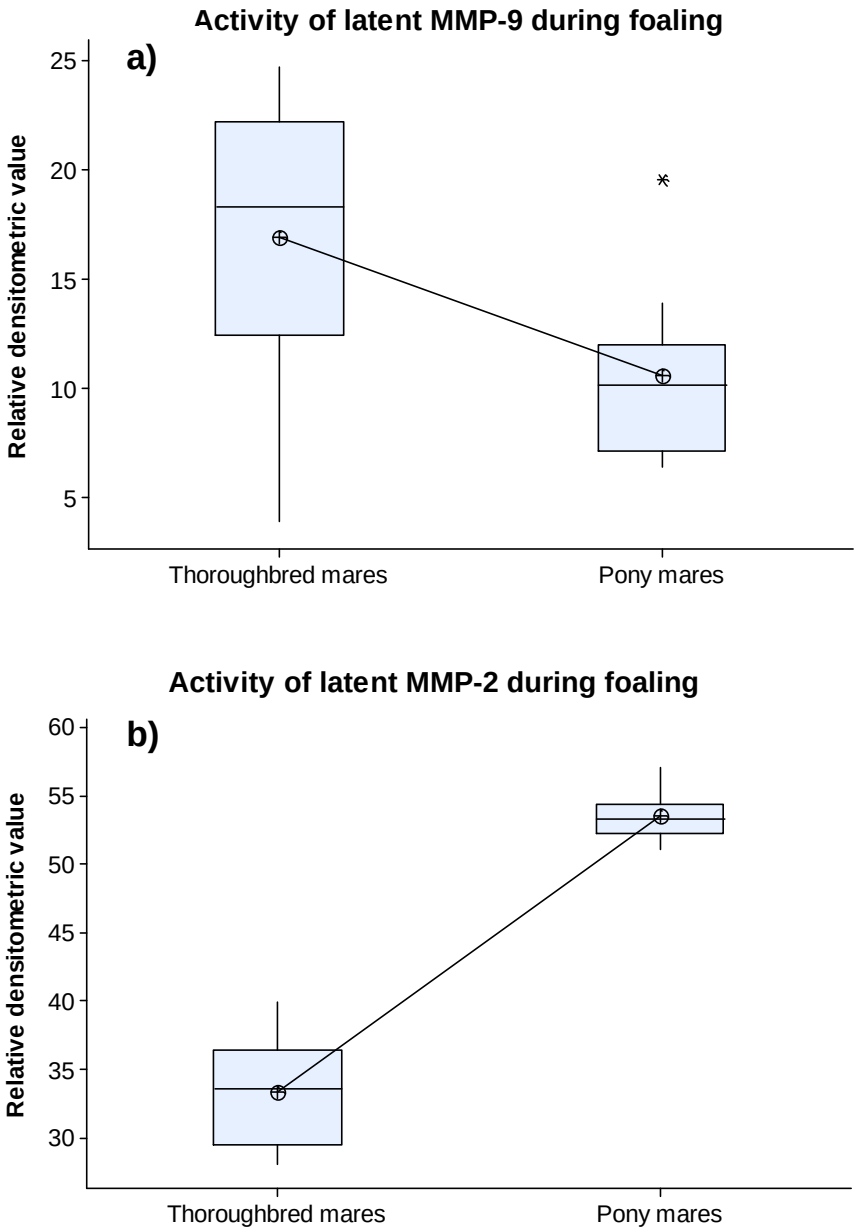


Figure 6.4 Activity of latent MMP-9 and MMP-2 during foaling in pony and thoroughbred mares Latent gelatinase MMP-9 and MMP-2 activity as demonstrated by gelatin zymography in equine amniotic flid. a) latent MMP-9 activity was statistically significantly (p=0.02) higher in thoroughbred mares than in pony mares. b) latent MMP-2 activity was statistically significantly higher (p<0.001) in pony mares than in thoroughbred mares.

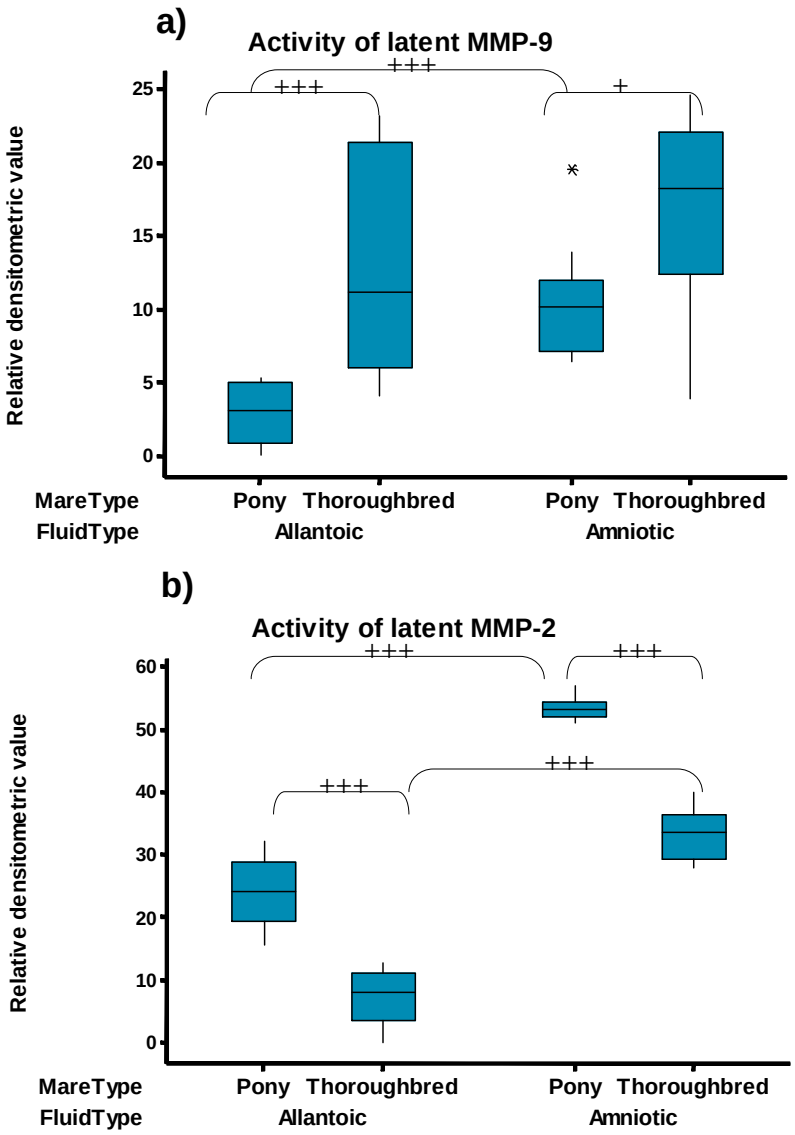


Figure 6.5 Activity of MMP-9 and MMP-2 in allantoic and amniotic fluid Latent MMP-9 a) and latent MMP-2 b) activies in foaling pony and thoroughbred mares. The trend for both MMPs is for less activity in allantoic than in amniotic fluid. Also, for both types of foetal fluid, latent MMP-2 activity was higher in pony mares and latent MMP-9 activity was higher in thoroughbred mares. +: $p<0.05$, +++: $p<0.001$.

6.2.3 MMP-2 and MMP-9 activities in allantoic and amniotic fluid

MMP-2 and MMP-9 activities were detected via zymography in the allantoic fluid samples taken from foaling pony and thoroughbred mares. When latent MMP-

2 (**Figure 6.5 b**) and latent MMP-9 (**Figure 6.5 a**) activities were compared between paired allantoic and amniotic fluids from foaling pony mares, activities of both enzymes were statistically significantly ($p<0.001$ for both) lower in allantoic fluid. In thoroughbred mares, latent MMP-9 (**Figure 6.5 a**) activity was not statistically significantly different ($p=0.14$) in allantoic fluid when compared to amniotic fluid, whereas latent MMP-2 activity (**Figure 6.5 b**) was statistically significantly ($p<0.001$) lower in allantoic fluid. When allantoic fluids were compared between foaling pony mares and thoroughbred mares, the activity of latent MMP-9 (**Figure 6.5 a**) was statistically significantly ($p<0.001$) higher in thoroughbreds than ponies, and latent MMP-2 (**Figure 6.5 b**) was statistically significantly ($p<0.001$) lower in thoroughbreds.

6.2.4 TIMP activity in amniotic fluid during gestation and foaling

The 21 kDa band corresponding to TIMP-2 was present in all samples analysed. No statistically significant difference ($p=0.20$) in activity was identified between mid-gestation and the pre-parturient period. There was no statistically significant difference ($p=0.14$) in TIMP-2 activity in normal pre-parturient samples and in samples taken during foaling in ponies. In pre-parturient samples (**Figure 6.6**), TIMP-2 activity was statistically significantly ($p<0.001$) higher in pregnancies resulting in normal foaling than in those resulting in abortions or stillbirths. Of all the TIMPs, only TIMP-2 was identified in amniotic fluids from foaling thoroughbred and pony mares, with no statistically significant ($p=0.25$) breed differences. The 27-30 and 24 kDa bands were detected in samples collected 1-3 days before normal foaling, but were absent in mid-gestation and 1-3 days before stillbirth or abortion.

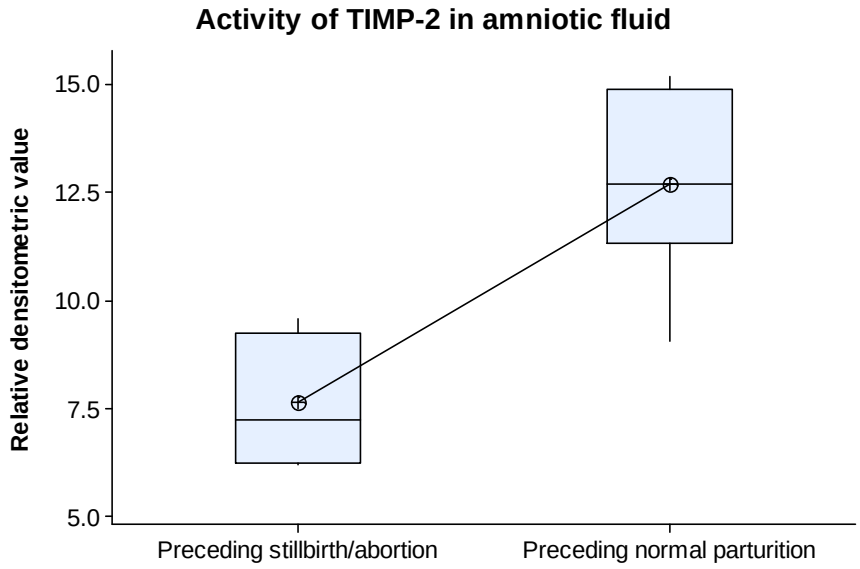


Figure 6.6 Activity of TIMP-2 in equine amniotic fluid during the pre-parturient period. The activity of TIMP-2 was statistically significantly ($p<0.001$) higher preceding normal parturition than preceding stillbirth or abortion.

6.3 Discussion

In this study it has been shown for the first time that MMP-2 and -9, as well as TIMP-2 are present in the allantoic and amniotic fluids during the latter half of equine pregnancy. The allantois of the equine foetus is unique, as it is not rudimentary as in most other species, but encloses and is in close apposition with the amniotic membrane as well as the chorion (Steven 1982). The equine allantois is extensive and increases in volume in the last trimester (Arthur 1969). Amniotic fluid in the mare is therefore likely to be different from human and ovine in source and composition, as it does not contain foetal urine but is composed of respiratory and oral secretions from the foetus, molecules from the maternal circulation and possibly secretions by cells in the allantoamnion (Asbury & LeBlanc 1993). Gelatin

zymography and reverse zymography demonstrated the activity of MMP-2 and MMP-9 as well as TIMP-2 in undiluted allantoic and amniotic fluids. This method reveals the absolute amounts of enzyme activity within these fluids without taking into account the dilution factor. In paired allantoic and amniotic fluid samples from foaling mares the activity of MMP-2 and MMP-9 was consistently less intense in allantoic than in amniotic fluids. The absolute difference in MMP activity between the two fluid compartments is likely to be a dilution effect to MMPs secreted into the foetal compartments. It is possible that cells of the chorioallantois and allantoamnion secrete MMPs into the foetal compartments, however due to the destructive capacity and precise regulation of these enzymes it is highly unlikely that they are secreted indiscriminently into a large volume of fluid.

Foetal cell populations likely to secrete MMP-2 and MMP-9 to promote tissue remodelling include the trophoblast cells of the chorioallantois and cells of the developing tissues in the foetal body such as the lung. Trophoblast cells have been identified as a source of both MMP-2 and MMP-9 in the ovine placenta (Riley *et al.* 2000), and this is likely to apply in the equine placenta also. The equine foetal lung undergoes growth and maturation essential for extrauterine survival late in gestation (Beech *et al.* 2001). Activities of MMP-9 and MMP-2 are not different between ovine foetal tracheal fluid and amniotic fluid, confirming the extensive communication between these compartments during foetal breathing movements (Devlieger *et al.* 2002). In this present study, dramatic increases were found after day 300 of gestation, a period when much of the foetal lung and surfactant maturation takes place (Pattle *et al.* 1975).

During the latter half of equine gestation considerable foetal and placental growth takes place, with increasing numbers and ongoing development of microplacentomes from day 105 of gestation until term (Macdonald *et al.* 2000a). The steady levels of MMP-9 and MMP-2 activities demonstrated from day 191 to 233 of gestation in this study indicate that some collagenolytic activity takes place, in agreement with ongoing increase in placental interdigitation.

The increase in amniotic latent MMP-9 activity in the pre-parturient period is likely to relate to the foetal and maternal events taking place in preparation for parturition. In a study on the ovine placenta, the activities of latent MMP-9 and active MMP-2 increased with advancing gestation and were still considerable during labour (Riley *et al.* 2000). The activity of MMP-9 in amniotic fluid has also been shown to increase during labour in humans, whereas MMP-2 activity is decreased (Maymon *et al.* 2000). These species differences may be due to different placental types and foetal compartments.

It has previously been shown that the activity of MMP-9, but not MMP-2, in ovine amniotic fluid increased during the healing of the foetal membranes after foetoscopic surgery (Devlieger *et al.* 2002). It is therefore possible that some of the MMP activity in the present study may result from repair processes in the foetal membranes following catheterization surgery. Uterine infection does not appear to have been induced by the surgical procedure, as most of the normal late pregnancies were sampled over a period of at least 7 days, and yielded healthy foals subsequently,

without clinical signs of intrauterine infection. Mid-pregnancy samples were collected over a minimum of 13 days and indicated a fluctuating pattern of MMP-9 activity, most increasing considerably for then to decrease as rapidly again, whereas the pattern in two mares was an initial decrease in activity. All of these mares also had normal foals at term. The amniotic activity of MMP-9 in most of the abortions and stillbirths did not increase approaching parturition in step with the normal pregnancies, indicating that an increase in MMP-9 activity is a normal physiologic event in late equine pregnancy.

Preparation for parturition of both foetus and dam takes place at this late stage in pregnancy. Both foetal membranes are likely to undergo remodelling resulting in reduced tensile strength as their rupture is required during foaling. In human foetal membranes a discrete area is programmed before birth to undergo ECM degradation to enable the initial rupture (McLaren *et al.* 2000) and a reduction in collagen bundles has been shown in rat amnion before parturition (Paavola *et al.* 1995). It is not known if similar remodelling takes place in the pre-parturient period in the mare or if the chorioallantoic rupture is primarily due to mechanical pressure during myometrial contractions. It has been reported that in the week approaching foaling, myometrial contractions increase up until 2-4 hours before delivery, but that in this period only minimal contractions take place. It is not until at the rupture of the chorioallantois that contractions gather momentum (Haluska *et al.* 1987). It is likely, therefore, that some weakening of the membrane does occur, as it has been reported that the membrane is resistant to mechanical rupture from day 90 of gestation (Allen & Bracher 1992).

Interestingly, the parturition of an aborted or stillborn foetus appeared to proceed without the preceding increase in latent MMP-9 activity, indicating that this enzyme is not essential for the rupture of foetal membranes. Placental separation does not normally occur until the foal is born, as is displayed by the fact that the outermost chorioallantois is normally delivered inside-out due to the late separation from the uterine wall. In this study, one of the stillborn foetuses was born within the chorioallantois in a 'red bag' delivery but the other six did not display abnormal chorioallantoic features. It was not indicated that the intact chorioallantois was due to the lack of MMP-9 activity in this one case. In the cow, MMP-2 and MMP-9 activity has a role in expulsion of the placenta, as their activity is altered in placentome homogenates from cows with retained placenta (Maj & Kankofer 1997). This will need further investigation in the mare, and an interesting focus could be to carry out a controlled study involving normal and 'red bag' deliveries, eventually to investigate MMP-9 as a marker in high risk pregnancies.

The activities of latent MMP-9 and MMP-2 were lower during foaling than in the last days before foaling. These samples were obtained from two groups of pony mares, and the foaling mares had not been catheterised. The initial samples from the chronically catheterised mares were taken at the time of catheterization surgery (6-25 days before foaling), before any tissue repair had taken place. In these samples activities of both MMPs were higher than in the foaling samples, indicating that this is a true difference. It may be that in the last day before foaling, the activities of MMP-9 and -2 are not required and they are therefore removed from the amniotic

fluid shortly before foaling. This is interesting, as it might be assumed that the foetal fluids served as 'pools' of surplus MMP molecules that were not actively removed from these fluids as this difference during foaling may indicate.

No difference was found in the pre-parturient period before normal births, and stillbirths or abortions in latent MMP-2 activity. Both groups had greater activity of latent MMP-2 in the last days before parturition than during active foaling. These results agree with a study on human amniotic fluid, where lower activity of MMP-2 was seen after rupture of foetal membranes, both at term and pre-term (Maymon *et al.* 2000). In another study, no difference was seen in MMP-2 activity between labour and non-labour human placental extracts (Xu *et al.* 2002). This difference is likely to be due to different samples and analytical methods used.

The activity of TIMPs has been previously described in human amniotic fluid, and all TIMPs were found to be decreased during active labour (Riley *et al.* 1999b). It was hypothesised that the coinciding increase in MMP-9 activity and reduction in the activity of TIMPs in amniotic fluids acted in unison to aid in membrane rupture during birth. In the present study, TIMP-2 activity remained relatively constant throughout normal late pregnancy and foaling, indicating that similar modulating mechanisms are not at work during equine parturition. On the other hand, the activities of the measured TIMPs were higher in pregnancies resulting in normal foaling than in those resulting in abortions or stillbirths, in keeping with the higher activity of MMP-9 seen preceding normal foaling.

Breed differences in MMP activities during foaling were demonstrated between pony and thoroughbred mares, as higher MMP-9 but lower MMP-2 activities were seen in thoroughbred mares. It has been shown that serum progestagen concentrations reach higher values during the pre-parturient period in thoroughbred mares than in pony mares (Allen *et al.* 2002a). Progestagens have a stimulatory effect on MMP-9 in human endometrium (Skinner *et al.* 1999), so this is a possible explanation for the higher activity of MMP-9 in thoroughbred amniotic fluid. It is uncertain if this difference in MMP activity has clinical relevance to the process of parturition in these breeds.

6.4 Conclusion

Both equine foetal compartments contain the latent gelatinases MMP-2 and MMP-9 that are likely to be secreted into the allantoic and amniotic fluid by placental components as well as the foetus. Allantoic fluid contains consistently less gelatinase activity than amniotic fluid that may simply be a result of dilution due to the greater volumes of allantoic fluid. This simple difference also indicates similar sources of MMPs into both fluids, although no direct physical communication exists to allow the fluids to communicate and equilibrate. During mid-gestation amniotic fluid activities of MMP-2 and MMP-9 fluctuate, but without a significant gestational trend. Approaching term the activity of latent MMP-9 increased during the last 25 days of gestation, possibly as a part of the preparation for ensuing parturition. This increase was not seen approaching abortions and stillbirths, although most of these parturitions took place without reported abnormalities in foetal membrane rupture or placental separation. Another likely source of increased activity of latent MMP-9 is

the foetal lung that undergoes essential and substantial tissue growth and maturation during this final stage of equine gestation. In amniotic fluids collected from foaling ponies the activity of MMP-9 was decreased when compared to pre-parturient values, and the activity was lower than in foaling thoroughbreds. MMP-2 activity on the other hand occurred without changes through mid- to late gestation, but during foaling the activity of the latent form was decreased and the active form increased. Both forms were less active in thoroughbred mares during foaling than foaling ponies. The activity of TIMP-2 was higher during the normal pre-parturient period than preceding abortions and stillbirths, and no breed differences were identified between thoroughbreds and ponies during foaling.

7 Chapter 7:

General discussion

7 General discussion

This thesis explored some of the mechanisms that have been extensively researched in connection with fibrosis of various organs, in order to investigate the pathogenesis of endometrial fibrosis in the mare. The aim of this thesis was to study the events involved in tissue remodelling occurring in the normal equine endometrium and how these events might be involved in the pathological deposition of collagen. The subject was approached by investigating the cellular and collagenolytic mechanisms during endometritis in the reproductively normal mare and by exploring various aetiological factors that might disrupt the normal pathways that are crucial to tissue remodelling. Additionally, tissue remodelling factors in the pregnant uterus were assessed. The main conclusions obtained from the results of this thesis are:

1. Tissue repair and remodelling occurring in the normal pregnant and non-pregnant equine endometrium as a result of endometritis involves cellular and enzymatic mechanisms similar to those involved in other organs and have been shown to be central to the development of fibrosis in these organs.

2. The study demonstrated that the secretion of MMP-2 and MMP-9 into the uterine lumen is minimal during the normal equine oestrous cycle, but is dramatically increased during endometritis. The activity of MMP-9 was demonstrated to reside in infiltrating eosinophils.

3. Endometrial fibrosis in the mare is likely to be influenced by genetic predisposition as shown by the positive correlation of endometrial collagen amount

and inbreeding coefficient of mares. The largely extensively bred population of Icelandic mares does not develop endometrial fibrosis to the same extent as mares bred under intensive management systems and endometrial degeneration does not appear to be as important in explaining subfertility in this population as it is in lighter, more intensively bred breeds.

4. The extensive placental and foetal growth occurring late in equine gestation involves the activities of MMP-2 and MMP-9, which are likely to contribute to the evolving foetal-maternal interface as well as the maturation process of foetal organs such as the lungs. During normal parturition, the activity of MMP-9 in amniotic fluid is sharply reduced from high pre-parturient levels. The activity of MMP-2 and MMP-9 is lower preceding pathological parturition than preceding normal parturition, indicating that these MMPs have a well defined role in the equine parturition process.

7.1 Cellular infiltrations during equine endometritis

Induced equine endometritis occurring as a result of natural breeding or bacterial infection can be considered as a model for the repair and remodelling events occurring regularly in the brood mare. In this study, it has been shown that this event involves an early recruitment of lymphocytes as well as neutrophils. Lymphocytes are key players in the adaptive immune response and participate in the antigen-specific response that is very prominent in mucosal tissues. Contrary to some previous observations, uterine lymphocytes appear to be recruited early on in the process. Lymphocytic infiltrations in endometrial tissue are therefore not always a

sign of chronic inflammation and might be due to a transient acute inflammation. Chronic lymphocytic infiltration is traditionally associated with decreased fertility in mares, whereas their transient presence in the endometrium should not permanently affect reproductive performance. Lymphocytes play a central role in stimulating cytokine secretion by resident endometrial cells and thereby induce the attraction of neutrophils and possibly eosinophils (reviewed by Kelly *et al.* 2002).

7.2 Indications of collagen remodelling events during endometritis

It was shown that the activities of MMP-2 and MMP-9 do not vary depending on the stage of the oestrous cycle and that they are greatly increased during equine endometritis. The regulation of MMP-9 is closely regulated, as increased secretion into the uterine lumen is seen rapidly and is of short duration. The amounts of MMP-9 secreted into the uterine lumen are extremely precise as was shown by limited individual variation in a small population sample of mares. This enzyme is as rapidly reduced in uterine contents, either by reduced secretion or the dilution by transudate, or by both mechanisms. Active MMP-9 was located in discrete cytoplasmic granules within eosinophils in endometrial sections, or in free granules in the stroma. Eosinophils, and especially degranulating eosinophils, have been associated by immunohistochemistry with the fibrotic portions of endometriosis in women (Blumenthal *et al.* 2000). It has been shown that eosinophils are a source of TGF-β and that they promote matrix production by human dermal fibroblasts (Birkland *et al.* 1994; Zhang *et al.* 1997). These cells are therefore a possible source of continuous activation of fibroblasts to secrete collagen resulting in fibrosis of the endometrium. It can therefore be deducted that the repair mechanisms in the

endometrium are likely to be the same as in the liver that is widely used as a model for the pathogenesis of fibrosis. Macrophages have also been shown to activate collagen deposition by myofibroblasts, but this cell population was beyond the scope of this study. However, the revealing results on the importance of eosinophils in endometrial remodelling warrant future detailed work on macrophages.

7.3 Aetiological factors correlated with endometrial fibrosis

An individual predisposition to endometrial fibrosis is known to occur in mares, as varying levels of endometrial fibrosis are frequently encountered in endometrial biopsies from mares of the same age. The positive correlation found between endometrial collagen and inbreeding coefficient of mares gives an insight into the mechanisms behind this individual predisposition. There is evidence of a genetic predisposition for fibrosis of the lung and liver, as a comparatively intense stimulus applied to different individuals can provoke a wide range of fibrotic responses. It is possible that the repair mechanisms responsible for maintaining a normally functioning endometrium are altered in mares with a genetic predisposition for the deposition of collagen. Increased levels of inbreeding lead to a decreased variety in the gene pool, with a raised risk of faulty genes prevailing. Clinically, it appears that endometrial degeneration occurs with increasing age, but a genetic predisposition may lead to an earlier onset of this condition. In the study population of Icelandic mares, inbreeding coefficients were low and this breed appears to develop less severe endometrial fibrosis than what is known from lighter breeds. It is therefore essential to carry out a comparable study using thoroughbred mares, to elucidate this relationship between endometrial fibrosis and inbreeding coefficient.

7.3.1 Endometrial fibrosis in Icelandic mares

Another important outcome from the study of Icelandic mares is the lack of relevance of the Kenney category system to this population. All mares were classified into the two intermediate categories, in spite of the inclusion in the study of mares with a history of reproductive problems, and a wide range of age groups. There was no correlation between the fertility of the mares, as defined by reproductive success in previous breeding seasons, and the condition of their endometrium, as defined by the Kenney category or the amount of collagen. Reproductive problems of Icelandic mares therefore do not appear to be explained by endometrial degeneration to the same extent as in other horse breeds.

7.3.2 MMP activity in established endometrial fibrosis

A correlation was not demonstrated between endometrial collagen deposition and the activities of MMP-2 and MMP-9 and TIMP-2 in uterine fluid in the study population of Icelandic mares. This result is unsurprising as the population was found to be very mildly affected by endometrial degeneration and therefore does not provide ideal study material for this condition. Furthermore, the result does not permanently exclude these enzymes from the proposed pathway leading to endometrial fibrosis, as they would not be expected to be present in constant amounts in the fibrotic uterus. It is therefore important to conduct a comparative study on an equine population known to develop severe endometrial fibrosis, and possibly to induce the action of MMPs in the fibrotic uterus to determine their activity in endometrial repair.

7.4 Indications of tissue remodelling during equine pregnancy

Pregnancy can be considered the normal physiological state of a reproductively mature mare and a protective effect of pregnancy has been proposed against endometrial degeneration (Chevalier-Clément 1989). In this study, it was shown that MMP-2 and MMP-9 are present in foetal fluids from mid-gestation until term. They are likely to be important in placental growth and interdigitation with the endometrium as well as the growth and maturation of foetal organs, such as the lungs. One plausible source of these MMPs during equine pregnancy are the trophoblast cells of the placenta. In a previous study the early invasive trophoblast cells of the equine chorionic girdle were demonstrated to digest a collagen matrix by secreting MMP-2 and MMP-9 (Vagnoni *et al.* 1995). The activity of MMP-9 increased steadily until term, but was significantly reduced in amniotic fluid collected during foaling. It appears that MMP-9 plays a role in specific uterine processes until term, but then is surplus to requirement and so secretion is discontinued very shortly before parturition. In contrast, the activity of MMP-2 remained at unchanging levels throughout gestation and was not altered in amniotic fluid collected during normal parturition. Curiously, the activity of MMP-2 was upregulated preceding stillbirths or abortions, accompanied by a reduction in TIMP-2 activity when compared to the normal pre-parturient period. It is difficult to ascertain the importance of these changes to the pathogenesis of abnormal parturition, and further investigation is needed to determine if this increase in activity is a reaction to an already occurring process or indeed the cause of it.

7.5 Can scar resolution be promoted in the endometrium?

Fibrosis in other organs, such as the liver, lung and kidney can be delayed or resolved by removing the cause of this reaction to tissue injury (reviewed by Franklin 1997). The removal of the stimulus responsible for continuous collagen deposition induces the scar resolution stage that should normally follow the repair process. During scar resolution the MMPs are induced to degrade excess collagen and therefore restore normal tissue structure. For this treatment approach to be relevant for endometrial fibrosis it is necessary to determine the stimulus that may lead to the continuous activation of collagen synthesis by myofibroblasts. Ultimately, the stimulus responsible for this continuous collagen synthesis is either external or part of the inherent cascade occurring during tissue repair.

It is possible that factors such as genetic predisposition alter the sensitivity of certain cells in the endometrium so that they overreact to stimuli that would otherwise be tolerated. This could explain the susceptibility of some mares to repeated spells of endometritis. Endometritis and endometrial fibrosis have previously been associated and each has been suggested as the cause of the other, however the cause of these conditions may well be part of the same complex and they might develop side by side. In allergic contact hypersensitivity, that is based on antigen recognition and attraction of other inflammatory cells by lymphocytes, the inflammatory reaction was shown to be prolonged in MMP-9 deficient mice, demonstrating the importance in this enzyme to the onset of tissue repair (Pilcher *et al.* 1999). The altered activity of MMP-9 in equine endometrial fibrosis may have a similar effect in affected mares by

leading to a constant inflammatory irritation of the endometrial tissue and therefore possibly an uncontrolled deposition of collagen.

7.6 Future directions

This thesis concentrated on the normal remodelling processes occurring in the equine endometrium and has demonstrated some parallels with the remodelling in other organs. In order to correlate these processes with the abnormal deposition of collagen in the endometrium future studies should be carried out on a population of mares with endometrial fibrosis. An endometritis induction model comparable to the one used in this study could be used to determine the reaction and regulation of MMP-2 and MMP-9 as an expression of endometrial repair mechanisms. Also, other MMPs could be investigated, such as interstitial collagenases and others involved in inflammation and repair. Due to the chronic nature of endometrial fibrosis, the ideal study for the investigation of the pathogenesis of this condition would be a longitudinal study monitoring a cohort of mares at regular intervals to observe the onset and development of endometrial fibrosis.

Results from this study point towards inbreeding as an important aetiological factor in the development of endometrial fibrosis. In a competitive breeding environment such as the thoroughbred industry targeted solely on racing performance, individuals with a strong desirable trait are likely to be used preferentially for breeding, thus reducing genetic variation. In a natural setting, subfertile mares would have a reduced likelihood of bringing their genes forward, whereas in an intensive breeding program, such bloodlines are artificially preserved. Whilst financial pressure will

invariably target specific parental traits, minimization of inbreeding should be the basis of the choice of sire in order to reduce negative effects on fertility.

8 References

Acland HM & Kenney RM 1983 Lesions of contagious equine metritis in mares. *Vet pathol* **20** 330-341.

Adler KB, Low RB, Leslie KO, Mitchell J & Evans JN 1989 Contractile cells in normal and fibrotic lung. *Lab invest* **60** 473-485.

Alexander CM, Howard EW, Bissell MJ & Werb Z 1996 Rescue of mammary epithelial cell apoptosis and entactin degradation by a tissue inhibitor of metalloproteinases-1 transgene. *J Cell Biol* **135** 1669-1677.

Alexander, P 2002 Extracellular matrix proteases & proteins technical guide. *Matrix metalloproteinases (MMPs) Calbiochem Merck* **2** 1-19.

Alghamdi AS, Foster DN & Troedsson MH 2004 Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri. *Reproduction* **127** 593-600.

Allen WR & Bracher V 1992 Videoendoscopic evaluation of the mare's uterus: III. Findings in the pregnant mare. *Equine Vet J* **24** 285-291.

Allen WR & Stewart F 2001 Equine placentation. *Reproduction, Fertility, & Development* **13** 623-634.

Allen WR, Wilsher S, Stewart F, Ousey J & Fowden A 2002a The influence of maternal size on placental, fetal and postnatal growth in the horse. II. Endocrinology of pregnancy. *J Endocrinol* **172** 237-246.

Allen WR, Wilsher S, Turnbull C, Stewart F, Ousey J, Rossdale PD & Fowden AL 2002b Influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development in utero. *Reproduction* **123** 445-453.

Amann RP & Pickett BW 1987 Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. *J Equine Vet Sci* **7** 145-173.

Amenábar JM, Martins GB, Cherubini K & Figueiredo MAZ 2006 Comparison between semi-automated segmentation and manual point-counting methods for quantitative analysis of histological sections. *J Oral Sci* **48** 139-143.

Anon. Terrestrial Animal Health Code 2006. Paris, OIE - World Organization for Animal Health.

Archer GT & Hirsch JG 1963 Isolation of granules from eosinophil leukocytes and study of their enzyme content. *J Exp Med* **118** 277-86.

Arosalo BM, Raekallio M, Rajamaki M, Holopainen E, Kastevaara T, Salonen H & Sankari S 2007 Detecting early kidney damage in horses with colic by measuring matrix metalloproteinase -9 and -2, other enzymes, urinary glucose and total proteins. *Acta Vet Scand* **49** 4.

Arthur GH 1969 The fetal fluids of domestic animals. *J Reprod Fertil Suppl* **9** 45-52.

Asbury AC, Halliwell RE, Foster GW & Longino SJ 1980 Immunoglobulins in uterine secretions of mares with differing resistance to endometritis. *Theriogenology* **14** 299-308.

Asbury AC & Hansen PJ 1987 Effects of susceptibility of mares to endometritis and stage of cycle on phagocytic activity of uterine-derived neutrophils. *J Reprod Fertil Suppl* **35** 311-316.

Asbury AC & LeBlanc MM 1993 The placenta. In *Equine Reproduction*, pp. 509-516. Eds AO McKinnon & JL Voss. Williams & Wilkins.

Asbury AC, Schultz KT, Klesius PH, Foster GW & Washburn SM 1982 Factors affecting phagocytosis of bacteria by neutrophils in the mare's uterus. *J Reprod Fertil Suppl* **32** 151-159.

Athayde N, Edwin SS, Romero R, Gomez R, Maymon E, Pacora P & Menon R 1998 A role for matrix metalloproteinase-9 in spontaneous rupture of the fetal membranes. *Am J Obstet Gynecol* **179** 1248-1253.

Auld DS 1995 Removal and replacement of metal ions in metallopeptidases. *Methods Enzymol* **248** 228-242.

Baker AH, Edwards DR & Murphy G 2002 Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci* **115** 3719-3727.

Barron JK 1995 The effect of maternal age and parity on the racing performance of Thoroughbred horses. *Equine Vet J* **27** 73-75.

Bataller R & Brenner DA 2005 Liver fibrosis. *J Clin Invest* **115** 209-218.

Bataller R, North KE & Brenner DA 2003 Genetic polymorphisms and the progression of liver fibrosis: a critical appraisal. *Hepatology* **37** 493-503.

Beech DJ, Sibbons PD, Rossdale PD, Ousey JC, Holdstock NB, Chavatte P & Ansari T 2001 Organogenesis of lung and kidney in Thoroughbreds and ponies. *Equine Vet J* **33** 438-445.

Betteridge KJ, Eaglesome MD, Mitchell D, Flood PF & Beriault R 1982 Development of horse embryos up to twenty-two days after ovulation: Observations on fresh specimens. *J Anat* **135** 191-209.

Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A & Engler JA 1993 Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* **4** 197-250.

Birkland TP, Cheavens MD & Pincus SH 1994 Human eosinophils stimulate DNA synthesis and matrix production in dermal fibroblasts. *Arch Dermatol Res* **158**.

Bitterman PB, Rennard SI, Keogh BA, Wewers MD, Adelberg S & Crystal RG 1986 Familial idiopathic pulmonary fibrosis. Evidence of lung inflammation in unaffected family members. *N Engl J Med* **314** 1343-1347.

Björklund M & Koivunen E 2005 Gelatinase-mediated migration and invasion of cancer cells. *Biochim Biophys Acta* **1755** 37-69.

Blue HB, Blue MG, Kenney RM & Merritt TL 1984 Chemotactic properties and protein of equine uterine fluid. *Am J Vet Res* **45** 1205-1208.

Blue MG, Brady AA, Davidson JN & Kenney RM 1982 Studies on the composition and antibacterial activity of uterine fluid from mares. *J Reprod Fertil Suppl* **32** 143-149.

Blumenthal RD, Samoszuk M, Taylor AP, Brown G, Alisauskas R & Goldenberg DM 2000 Degranulating eosinophils in human endometriosis. *Am J Pathol* **156** 1581-1588.

Boos A, Janssen V & Mulling C 2003 Proliferation and apoptosis in bovine placentomes during pregnancy and around induced and spontaneous parturition as well as in cows retaining the fetal membranes. *Reproduction* **126** 469-480.

Borregaard N 1997 Development of neutrophil granule diversity. *Ann N Y Acad Sci* **832** 62-68.

Bracher, V 1997 Influence of age and parity on the equine endometrium. *Pferdeheilkunde* **13** 549.

Bracher V & Allen WR 1992 Videoendoscopic evaluation of the mare's uterus: I. Findings in normal fertile mares. *Equine Vet J* **24** 274-278.

Bracher V, Mathias S & Allen WR 1992 Videoendoscopic evaluation of the mare's uterus: II. Findings in subfertile mares. *Equine Vet J* **24** 279-284.

Bracher V, Mathias S & Allen WR 1996 Influence of chronic degenerative endometritis (endometrosis) on placental development in the mare. *Equine Vet J* **28** 180-188.

Brandtzaeg P 1997 Mucosal immunity in the female genital tract. *J Reprod Immunol* **36** 23-50.

Bristol F 1987 Fertility of pasture bred mares in synchronised oestrus. *J Reprod Fert Suppl* **35** 39-43.

Brook D 1993 Uterine cytology. In *Equine Reproduction,* pp. 246-253. Eds AO McKinnon & JL Voss. Williams & Wilkins.

Cadario ME, Losinno L, Giguere S, Aguilar J, Jack T J, Macpherson M, Fitzpatrick C & Uhl EW 2002 Uterine expression of fibrogenic cytokines in the mare. *Theriogenology* **58** 449-452

Cadario ME, Thatcher WW, Klapstein E, Merrit AM, Archbald LF, Thatcher MJ & LeBlanc MM 1999 Dynamics of prostaglandin secretion, intrauterine fluid and uterine clearance in reproductively normal mares and mares with delayed uterine clearance. *Theriogenology* **52** 1181-1192.

Capraro DL, Vernon MW, Abrams RM & Sharp DC 1976 Effects of oxytocin and $PGF_2\alpha$ on mare uterine motility. *J Anim Sci* **43** 277.

Card C 2005 Post-breeding inflammation and endometrial cytology in mares. *Theriogenology* **64** 580-588.

Carlson MA & Longaker MT 2004 The fibroblast-populated collagen matrix as a model of wound healing: a review of the evidence. *Wound Repair Regen* **12** 134-147.

Carnevale EM, Coutinho da Silva MA, Panzani D, Stokes JE & Squires EL 2005 Factors affecting the success of oocyte transfer in a clinical program for subfertile mares. *Theriogenology* **64** 519-527.

Carnevale EM & Ginther OJ 1992 Relationships of age to uterine function and reproductive efficiency in mares. *Theriogenology* **37** 1101-1115.

Carnevale EM, Ramirez RJ, Squires EL, Alvarenga MA, Vanderwall DK & McCue PM 2000 Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. *Theriogenology* **54** 965-979.

Chaudhuri V & Karasek MA 2006 Mechanisms of microvascular wound repair II. Injury induces transformation of endothelial cells into myofibroblasts and the synthesis of matrix proteins. *In Vitro Cell Dev Biol Anim* **42** 314-9.

Chen EI, Li W, Godzik A, Howard EW & Smith JW 2003 A residue in the S2 subsite controls substrate selectivity of matrix metalloproteinase-2 and matrix metalloproteinase-9. *J Biol Chem* **278** 17158-17163.

Cheung AT, Liu IK, Walsh EM & Miller ME 1985 Phagocytic and killing capacities of uterine-derived polymorphonuclear leukocytes from mares resistant and susceptible to chronic endometritis. *Am J Vet Res* **46** 1938-1940.

Chevalier-Clément F 1989 Pregnancy loss in the mare. *Animal Reprod Sci* **20** 231-244.

Chu Py PY, Salamonsen LA, Lee CS & Wright PJ 2002 Matrix metalloproteinases (MMPs) in the endometrium of bitches. *Reproduction* **123** 467-477.

Clegg PD, Burke RM, Coughlan AR, Riggs CM & Carter SD 1997a Characterisation of equine matrix metalloproteinase 2 and 9; and identification of the cellular sources of these enzymes in joints. *Equine Vet J* **29** 335-342.

Clegg PD & Carter SD 1999 Matrix metalloproteinase-2 and -9 are activated in joint diseases. *Equine Vet J* **31** 324-330.

Clegg PD, Coughlan AR, Riggs CM & Carter SD 1997b Matrix metalloproteinases 2 and 9 in equine synovial fluids. *Equine Vet J* **29** 343-348.

Collier IE, Saffarian S, Marmer BL, Elson EL & Goldberg G 2001 Substrate recognition by gelatinase A: the C-terminal domain facilitates surface diffusion. *Biophys J* **81** 2370-2377.

Corcoran ML & Stetler-Stevenson WG 1995 Tissue inhibitor of metalloproteinase-2 stimulates fibroblast proliferation via a cAMP-dependent mechanism. *J Biol Chem* **270** 13453-13459.

Crossett B, Allen WR & Stewart F 1996 A 19 kDa protein secreted by the endometrium of the mare is a novel member of the lipocalin family. *Biochem J* **320** 137-143.

Curry TE, Jr. & Osteen KG 2003 The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocr Rev* **24** 428-465.

Darenius K, Kindahl H & Madej A 1988 Clinical and endocrine studies in mares with known history of repeated conceptus losses. *Theriogenology* **29** 1215-1232.

Davies Morel MC & Gunnarsson V 2000 A survey of the fertility of Icelandic stallions. *Animal Reprod Sci* **64** 49-64.

Davis SD, Grubaugh WR & Weithenauer J 1987 Follicle integrity and serum estradiol 17β patterns during sexual recrdescence in the mare. *Biol Reprod Suppl* **36** 121.

Devlieger R, Riley SC, Verbist L, Leask R, Pijnenborg R & Deprest JA 2002 Matrix metalloproteinases-2 and -9 and their endogenous tissue inhibitors in tissue remodeling after sealing of the fetal membranes in a sheep model of fetoscopic surgery. *J Soc Gynecol Invest* **9** 137-145.

Dixon SN & Gibbons RA 1979 Proteins in the uterine secretions of the cow. *J Reprod Fertil* **56** 119-127.

Doig PA, McKnight JD & Miller RB 1981 The use of endometrial biopsy in the infertile mare. *Can Vet J* **22** 72-76.

Doig PA & Waelchli RO 1993 Endometrial biopsy. In *Equine Reproduction*, pp. 225-233. Eds AO McKinnon & JL Voss. Philadelphia: Williams & Wilkins.

Douglas-Hamilton DH, Osol R, Osol G, Driscoll D & Noble H 1984 A field study of the fertility of transported equine semen. *Theriogenology* **22** 291-304.

Dunkel B, Dolente B & Boston RC 2005 Acute lung injury/acute respiratory distress syndrome in 15 foals. *Equine Vet J* **37** 435-440.

Edwards DR, Leco KJ, Beaudry PP, Atadja PW, Veillette C & Riabowol KT 1996 Differential effects of transforming growth factor-beta 1 on the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in young and old human fibroblasts. *Exp Gerontol* **31** 207-223.

el Nahas AM, Muchaneta-Kubara EC, Essawy M & Soylemezoglu O 1997 Renal fibrosis: insights into pathogenesis and treatment. *Int J Biochem Cell Biol* **29** 55-62.

Evans MJ, Hamer JM, Gason LM, Graham CS, Asbury AC & Irvine CH 1986 Clearance of bacteria and non-antigenic markers following intra-uterine inoculation into maiden mares: Effect of steroid hormone environment. *Theriogenology* **26** 37-50.

Evans MJ, Hamer JM, Gason LM & Irvine CHG 1987 Factors affecting uterine clearance of inoculated materials in mares. *J Reprod Fertil Suppl* **35** 327-334.

Evans TJ, Miller MA, Ganjam VK, Niswender KD, Ellersieck MR, Krause WJ & Youngquist RS 1998 Morphometric analysis of endometrial periglandular fibrosis in mares. *Am J Vet Res* **59** 1209-1214.

Fata JE, Ho AT, Leco KJ, Moorehead RA & Khokha R 2000 Cellular turnover and extracellular matrix remodeling in female reproductive tissues: functions of metalloproteinases and their inhibitors. *Cell Mol Life Sci* **57** 77-95.

Ferreira-Dias G, Nequin LG & King SS 1994 Morphologic characteristics of equine endometrium classified as Kenney categories I, II, and III, using light and scanning electron microscopy. *Am J Vet Res* **55** 1060-1065.

Ferreira-Dias GM, Nequin LG & King SS 1999 Morphologic comparisons among equine endometrium categories I, II, and III, using light and transmission electron microscopy. *Am J Vet Res* **60** 49-55.

Finn CA 1986 Implantation, menstruation and inflammation. *Biol Rev Camb Philos Soc* **61** 313-328.

Flood PF 1991 The development of the conceptus and its relationship to the uterus. In *Reproduction in domestic animals*, pp. 315-360. Ed PT Cupps. London: Academic Press.

Flood PF, Betteridge KJ & Diocee MS 1982 Transmission electron microscopy of horse embryos 3-16 days after ovulation. *J Reprod Fertil Suppl* **32** 319-27.

Frank MM & Fries LF 1991 The role of complement in inflammation and phagocytosis. *Immunol Today* **12** 322-326.

Franklin TJ 1997 Therapeutic approaches to organ fibrosis. *Int J Biochem Cell Biol* **29** 79-89.

Fumuso E, Giguere S, Wade J, Rogan D, Videla-Dorna I & Bowden RA 2003 Endometrial IL-1beta, IL-6 and TNF-alpha, mRNA expression in mares resistant or susceptible to post-breeding endometritis. Effects of estrous cycle, artificial insemination and immunomodulation. *Vet Immunol Immunopathol* **96** 31-41.

Ganjam VK, McLeod C, Klesius PH, Washburn SM, Kwapien R, Brown B & Fazeli MH 1982 Effect of ovarian hormones on the phagocytic response of ovariectomised mares. *J Reprod Fertil Suppl* **32** 169-174.

Gerstenberg C, Allen WR & Stewart F 1999 Cell proliferation patterns during development of the equine placenta. *J Reprod Fertil* **117** 143-152.

Ginther OJ 1983a Mobility of the early equine conceptus. *Theriogenology* **19** 603-611.

Ginther OJ 1983b Fixation and orientation of the early equine conceptus. *Theriogenology* **19** 613-623.

Godkin JD & Doré JJ 1998 Transforming growth factor beta and the endometrium. *Rev Reprod* **3** 1-6.

Goffin F, Munaut C, Frankenne F, Perrier D'Hauterive S, Béliard A, Fridman V, Nervo P, Colige A & Foidart JM 2003 Expression pattern of metalloproteinases and tissue inhibitors of matrix-metalloproteinases in cycling human endometrium. *Biol Reprod* **69** 976-84.

Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm S & He CS 1989 Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2. *Proc Natl Acad Sci USA* **86** 8207-8211.

Goldman S, Weiss A, Eyali V & Shalev E 2003 Differential activity of the gelatinases (matrix metalloproteinases 2 and 9) in the fetal membranes and decidua, associated with labour. *Mol Hum Reprod* **9** 367-373.

Gray CA, Bartol FF, Tarleton BJ, Wiley AA, Johnson GA, Bazer FW & Spencer TE 2001 Developmental biology of uterine glands. *Biol Reprod* **65** 1311-1323.

Greiling D & Clark RA 1997 Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix. *J Cell Sci* **110** 861-870.

Gressner AM, Weiskirchen R, Breitkopf K & Dooley S 2002 Roles of TGF-beta in hepatic fibrosis. *Front Biosci* **7** d793-d807.

Güvenc K, Reilas T & Katila T 2004 Effect of frozen semen on the uterus of mares with pathological uterine changes. *Reprod Nutr Dev* **44** 243-50.

Håkansson A, Albihn A & Magnusson U 1993 The contribution of complement to opsonic activity in the uterine secretions of mares free of endometritis. *Theriogenology* **39** 601-609.

Haluska GJ, Lowe JE & Currie WB 1987 Electromyographic properties of the myometrium correlated with the endocrinology of the pre-partum and post-partum periods and parturition in pony mares. *J Reprod Fertil Suppl* **35** 553-564.

Heiskanen ML, Huhtinen M, Pirhonen A & Mäenpää PH 1994 Insemination results with slow-cooled stallion semen stored for 70 or 80 hours. *Theriogenology* **42** 1043-51.

Henriet P, Cornet PB, Lemoine P, Galant C, Singer CF, Courtoy PJ, Eeckhout Y & Marbaix E 2002 Circulating ovarian steroids and endometrial matrix metalloproteinases (MMPs). *Ann N Y Acad Sci* **955** 119-138.

Heussen C & Dowdle EB 1980 Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerised substrates. *Anal Biochem* **102** 196-202.

Hinrichs K, Cummings MR, Sertich PL & Kenney RM 1-7-1988 Clinical significance of aerobic bacterial flora of the uterus, vagina, vestibule, and clitoral fossa of clinically normal mares. *J Am Vet Med Assoc* **193** 72-75.

Hipps DS, Hembry RM, Docherty AJ, Reynolds JJ & Murphy G 1991 Purification and characterization of human 72-kDa gelatinase (type IV collagenase). Use of immunolocalisation to demonstrate the non-coordinate regulation of the 72-kDa and 95-kDa gelatinases by human fibroblasts. *Biol Chem Hoppe Seyler* **372** 287-296.

Hu J, Van den Steen PE, Sang QX & Opdenakker G 2007 Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat Rev Drug Discov* **6** 480-498.

Hugason K, Árnason T & Jónmundsson JV 1985 A note on the fertility and some demographical parameters of Icelandic Toelter horses. *Livest Prod Sci* **12** 161-167.

Hughes JP & Loy RG 1969 Investigations on the effect of intrauterine inoculation of *Streptococcus zooepidemicus* in the mare. *Proc Am Ass Equine Pract* **51** 289-292.

Hulboy DL, Rudolph LA & Matrisian LM 1997 Matrix metalloproteinases as mediators of reproductive function. *Mol Hum Reprod* **3** 27-45.

Huppertz B, Kertschanska S, Demir AY, Frank HG & Kaufmann P 1998 Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. *Cell Tissue Res* **291** 133-148.

Iredale JP 1997 Tissue inhibitors of metalloproteinases in liver fibrosis. *Int J Biochem Cell Biol* **29** 43-54.

Jeffcott LB, Rossdale PD, Freestone J, Frank CJ & Towers-Clark PF 1982 An assessment of wastage in Thoroughbred racing from conception to 4 years of age. *Equine Vet J* **14** 185-198.

Jeffcott LB & Whitwell KE 1973 Twinning as a cause of foetal and neonatal loss in the thoroughbred mare. *J Comp Pathol* **83** 91-106.

Jones CL, Fecondo J, Kelynack K, Forbes J, Walker R & Becker G 1995 Tissue inhibitor of the metalloproteinases and renal extracellular matrix accumulation. *Exp Nephrol* **3** 80-86.

Jones DM, Fielden ED, Carr DH, Wade JE, Allen WR, Rossdale PD & Rowlands IW 1991 Some physiological and pharmacological factors affecting uterine motility as measured by electromyography in the mare. *J Reprod Fertil Suppl* **44** 357-368.

Jones RL, Squires EL, Slade NP, Pickett BW & Voss JL 1984 The effect of washing on the aerobic bacterial flora of the stallion's penis. *Proceedings of the American Association of Equine Practitioners* **30** 9-16.

Junqueira LCU 1979 Picro-sirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J* **11** 447-455.

Kaplanski G, Marin V, Montero-Julian F, Mantovani A & Farnarier C 2003 IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends Immunol* **24** 25-29.

Katila T 1995 Onset and duration of uterine inflammatory response of mares after insemination with fresh semen. *Biol Reprod Monogr* **1** 515-517.

Katila T 2005 Effect of the inseminate and the site of insemination on the uterus and pregnancy rates of mares. *Anim Reprod Sci* **89** 31-38.

Keenan LR, Forde D, McGeady T, Wade J & Roche JF 1987 Endometrial histology of early pregnant and non-pregnant mares. *J Reprod Fertil Suppl* **35** 499-504.

Kelly RW, King AE & Critchley HO 2001 Cytokine control in human endometrium. *Reproduction* **121** 3-19.

Kelly RW, King AE & Critchley HO 2002 Inflammatory mediators and endometrial function--focus on the perivascular cell. *J Reprod Immunol* **57** 81-93.

Kenney RM 1978 Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. *J Am Vet Med Ass* **172** 241-262.

Kenney RM & Doig PA 1986 Equine endometrial biopsy. In *Current Therapy in Theriogenology*, p. 723. Ed DA Morrow. Philadelphia: W.B. Saunders.

Kim H, Oda T, Lopez-Guisa J, Wing D, Edwards DR, Soloway PD & Eddy AA 2001 TIMP-1 deficiency does not attenuate interstitial fibrosis in obstructive nephropathy. *J Am Soc Nephrol* **12** 736-748.

King AE, Fleming DC, Critchley HO & Kelly RW 2003 Differential expression of the natural antimicrobials, beta-defensins 3 and 4, in human endometrium. *J Reprod Immunol* **59** 1-16.

Kolm G, Gemeiner M, Deichsel K, Budik S, Aurich J & Aurich C 2005 Effect of clenbuterol on the clearance of particles of charcoal (4 to 90 µm) from the uteri of mares. *Vet Rec* **156** 279-281.

Kotilainen T, Huhtinen M & Katila T 1994 Sperm-induced leukocytosis in the equine uterus. *Theriogenology* **41** 629-636.

Lacraz S, Nicod LP, Chicheportiche R, Welgus HG & Dayer JM 1995 IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. *J Clin Invest* **96** 2304-2310.

Lathbury LJ & Salamonsen LA 2000 In-vitro studies of the potential role of neutrophils in the process of menstruation. *Mol Hum Reprod* **6** 899-906.

Lea RG & Bolton AE 1991 The effect of horse placental tissue extracts and equine chorionic gonadotrophin on the proliferation of horse lymphocytes stimulated in vitro. *J Reprod Immunol* **19** 13-23.

LeBlanc MM, Asbury AC & Lyle SK 1989 Uterine clearance mechanisms during the early postovulatory period in mares. *Am J Vet Res* **50** 864-867.

Leishman D, Miller RB & Doig PA 1982 A quantitative study of the histological morphology of the endometrium of normal and barren mares. *Can J Comp Med* **46** 17-20.

Leiva MC, Xu Q, Galman M & Lyttle CR 1991 Ontogeny of the production of an estrogen-regulated eosinophil chemotactic factor in the rat uterus. *Biol Reprod* **45** 818-823.

Locksmith G, Clark P, Duff P, Saade G & Schultz G 2001 Amniotic fluid concentrations of matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1 during pregnancy and labor. *Am J Obstet Gynecol* **184** 159-164.

Lopata A, Agresta F, Quinn MA, Smith C, Ostor AG & Salamonsen LA 2003 Detection of endometrial cancer by determination of matrix metalloproteinases in the uterine cavity. *Gynecol Oncol* **90** 318-324.

Lorena D, Uchio K, Costa AM & Desmouliere A 2002 Normal scarring: importance of myofibroblasts. *Wound Repair Regen* **10** 86-92.

Lukashev ME & Werb Z 1998 ECM signalling: orchestrating cell behaviour and misbehaviour. *Trends Cell Biol* **8** 437-441.

Lunn P, Vagnoni KE & Ginther OJ 1997 The equine immune response to endometrial cups. *J Reprod Immunol* **34** 203-216.

Macdonald AA, Chavatte P & Fowden AL 2000 Scanning electron microscopy of the microcotyledonary placenta of the horse (Equus caballus) in the latter half of gestation. *Placenta* **21** 565-574.

Macdonald AA & Fowden AL 1997 Microscopic anatomy of the ungulate placenta. *Equine Vet J Suppl* 7-13.

MacIntyre N 2001 Unmasking antigens for immunohistochemistry. *Br J Biomed Sci* **58** 190-196.

Maj JG & Kankofer M 1997 Activity of 72-kDa and 92-kDa matrix metalloproteinases in placental tissues of cows with and without retained fetal membranes. *Placenta* **18** 683-687.

Malle E, Furtmuller PG, Sattler W & Obinger C 2007 Myeloperoxidase: a target for new drug development? *Br J Pharmacol* **152** 838-54

Maloufi F, Pierson RA, Otto S, Ball C, and Card C 2002 Mares susceptible or resistant to endometritis have similar endometrial echographic and inflammatory cell reactions at 96 hours after infusion with frozen semen and extender. *Proc Am Ass Equine Pract* **48** 51-57.

Mankhey RW, Wells CC, Bhatti F & Maric C 2007 17ß-estradiol supplementation reduces tubulointerstitial fibrosis by increasing MMP activity in the diabetic kidney. *Am J Physiol Regul Integr Comp Physiol* **292** R769-R777.

Mansour GD, Henry M & Ferreira AM 2003 Immunohistochemical study of equine endometrial extracellular matrix during the oestrous cycle. *J Comp Pathol* **129** 316-319.

Marra F 1999 Hepatic stellate cells and the regulation of liver inflammation. *J Hepatol* **31** 1120-1130.

Marshall RP, McAnulty RJ & Laurent GJ 1997 The pathogenesis of pulmonary fibrosis: is there a fibrosis gene? *Int J Biochem Cell Biol* **29** 107-120.

Martin RG, Penhale WJ & Williamson P 1988 Factors affecting the composition of mare uterine fluid. *Res Vet Sci* **45** 111-116.

Mauch C, Krieg T & Bauer EA 1994 Role of the extracellular matrix in the degradation of connective tissue. *Arch Dermatol Res* **287** 107-114.

Maymon E, Romero R, Pacora P, Gervasi MT, Gomez R, Edwin SS & Yoon BH 2000 Evidence of in vivo differential bioavailability of the active forms of matrix metalloproteinases 9 and 2 in parturition, spontaneous rupture of membranes, and intra-amniotic infection. *Am J Obstet Gynecol* **183** 887-894.

McAnulty RJ, Hernandez-Rodriguez NA, Mutsaers SE, Coker RK & Laurent GJ 1997 Indometacin suppresses the anti-proliferative effects of transforming growth factor-beta isoforms on fibroblast cell cultures. *Biochem J* **321** 639-643.

McDonnell AM & Watson ED 1992 The effect of transcervical uterine manipulations on establishment of uterine infection in mares under the influence of progesterone. *Theriogenology* **38** 945-950.

McKinnon AO, Voss JL, Squires EL & Carnevale EM 1993 Diagnostic ultrasonography. In *Equine Reproduction*, pp. 266-302. Eds AO McKinnon & JL Voss. Williams & Wilkins.

McLaren J, Taylor D & Bell S 2000 Increased concentration of pro-matrix metalloproteinase 9 in term fetal membranes overlying the cervix before labor: Implications for membrane remodeling and rupture. *Am J Obstet Gynecol* **182** 409-416.

Mercer JE, Clements JA, Funder JW & Clarke IJ 1988 Luteinizing hormone-beta mRNA levels are regulated primarily by gonadotropin-releasing hormone and not by negative estrogen feedback on the pituitary. *Neuroendocrinology* **47** 563-6.

Milani S, Herbst H, Schuppan D, Surrenti C, Riecken EO & Stein H 1990 Cellular localization of type I III and IV procollagen gene transcripts in normal and fibrotic human liver. *Am J Pathol.* **137** 59-70.

Mitchell G, Liu IK, Perryman LE, Stabenfeldt GH & Hughes JP 1982 Preferential production and secretion of immunoglobulins by the equine endometrium--a mucosal immune system. *J Reprod Fertil Suppl* **32** 161-168.

Morris LH & Allen WR 2002 Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. *Equine Vet J* **34** 51-60.

Mulayim N, Savlu A, Guzeloglu-Kayisli O, Kayisli UA & Arici A 2004 Regulation of endometrial stromal cell matrix metalloproteinase activity and invasiveness by interleukin-8. *Fertil Steril* **81 Suppl 1** 904-911.

Müller Z 1982 Fertility of frozen equine semen. *J Reprod Fertil Suppl* **32** 47-51.

Mutsaers SE, Bishop JE, McGrouther G & Laurent GJ 1997 Mechanisms of tissue repair: from wound healing to fibrosis. *Int JBiochem Cell Biol* **29** 5-17.

Nagase H, Visse R & Murphy G 2006 Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res* **69** 562-573.

Nikolakopoulos E, Kindahl H & Watson ED 2000 Oxytocin and PGF2(alpha) release in mares resistant and susceptible to persistent mating-induced endometritis. *J Reprod Fertil Suppl* **56** 363-372.

Nikolakopoulos E & Watson ED 1997 Does artificial insemination with chilled, extended semen reduce the antigenic challenge to the mare's uterus compared with natural service? *Theriogenology* **47** 583-590.

Nikolakopoulos E & Watson ED 1999 Uterine contractility is necessary for the clearance of intrauterine fluid but not bacteria after bacterial infusion in the mare. *Theriogenology* **52** 413-423.

Olsen LM, Al-Bagdadi FK, Richardson GF, Archbald LF, Braun WF, McCoy DJ, Godke RA, Titkemeyer CW & Thompson DL 1992 A histological study of the effect of saline and povidone-iodine infusions on the equine endometrium. *Theriogenology* **37** 1311-1325.

Olson MW, Bernardo MM, Pietila M, Gervasi DC, Toth M, Kotra LP, Massova I, Mobashery S & Fridman R 2000 Characterization of the monomeric and dimeric forms of latent and active matrix metalloproteinase-9. Differential rates for activation by stromelysin 1. *J Biol Chem* **275** 2661-2668.

Olson MW, Gervasi DC, Mobashery S & Fridman R 1997 Kinetic analysis of the binding of human matrix metalloproteinase-2 and -9 to tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. *J Biol Chem* **272** 29975-29983.

Ousey JC, Forhead AJ, Rossdale PD, Grainger L, Houghton E & Fowden AL 2003 Ontogeny of uteroplacental progestagen production in pregnant mares during the second half of gestation. *Biol Reprod* **69** 540-548.

Overall CM, Wrana JL & Sodek J 1991 Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. *J Biol Chem* **266** 14064-14071.

Paavola LG, Furth EE, Delgado V, Boyd CO, Jacobs CC, Lei H & Strauss JF, III 1995 Striking changes in the structure and organization of rat fetal membranes precede parturition. *Biol Reprod* **53** 321-338.

Pattle RE, Rossdale PD, Schock C & Creasey JM 1975 The development of the lung and its surfactant in the foal and in other species. *J Reprod Fertil Suppl* **23** 651-657.

Peterson FB, McFeely RA & David JSE 1969 Studies on the pathogenesis of endometritis in the mare. *Proc Am Ass Equine Pract* **51** 279-282.

Phan SH 2002 The myofibroblast in pulmonary fibrosis. *Chest* **122** 286S-289S.

Pickett BW 1993 Seminal extenders and cooled semen. In *Equine Reproduction*, pp. 746-753. Eds AO McKinnon & JL Voss. Philadelphia: Williams & Wilkins.

Pilcher BK, Wang M, Qin XJ, Parks WC, Senior RM & Welgus HG 1999 Role of matrix metalloproteinases and their inhibition in cutaneous wound healing and allergic contact hypersensitivity. *Ann N Y Acad Sci* **878** 12-24.

Pinheiro JC & Bates DM 2000 Mixed-effects models. In *S and S-plus* New York: Springer-Verlag.

Polette M, Nawrocki B, Pintiaux A, Massenat C, Maquoi E, Volders L, Schaaps JP, Birembaut P & Foidart JM 1994 Expression of gelatinases A and B and their tissue inhibitors by cells of early and term human placenta and gestational endometrium. *Lab Invest* **71** 838-846.

Portus BJ, Reilas T & Katila T 2005 Effect of seminal plasma on uterine inflammation, contractility and pregnancy rates in mares. *Equine Vet J* **37** 515-9.

Pycock JF & Allen WE 1990 Inflammatory components in uterine fluid from mares with experimentally induced bacterial endometritis. *Equine Vet J* **22** 422-425.

Qin X, Chua PK, Ohira RH & Bryant-Greenwood GD 1997a An autocrine/paracrine role of human decidual relaxin. II. Stromelysin-1 (MMP-3) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). *Biol Reprod* **56** 812-820.

Qin X, Garibay-Tupas J, Chua PK, Cachola L & Bryant-Greenwood GD 1997b An autocrine/paracrine role of human decidual relaxin. I. Interstitial collagenase (matrix metalloproteinase-1) and tissue plasminogen activator. *Biol Reprod* **56** 800-811.

Ratnikov B, Deryugina E, Leng J, Marchenko G, Dembrow D & Strongin A 2000 Determination of matrix metalloproteinase activity using biotinylated gelatin. *Anal Biochem* **286** 149-155.

Raulo SM 2001 Matrix metalloproteinases as markers of inflammation in equine chronic obstructive pulmonary disease (COPD) with special references to gelatinases and collagenases, Department of Veterinary Clinical Sciences, Faculty of Veterinary Medicine, University of Helsinki.

Reilas T & Katila T 2002 Proteins and Enzymes in Uterine Lavage Fluid of Postpartum and Nonparturient Mares. *Reprod Domest Anim* **37** 261-268.

Reilas T, Katila T, Makela O, Huhtinen M & Koskinen E 1997 Intrauterine fluid accumulation in oestrous mares. *Acta Vet Scand* **38** 69-78.

Reiswig JD, Threlfall WR & Rosol TJ 1993 A comparison of endometrial biopsy, culture and cytology during oestrus and dioestrus in the horse. *Equine Vet J* **25** 240-241.

Ricketts SW 1975a The technique and clinical application of endometrial biopsy in the mare. *Equine Vet J* **7** 102-108.

Ricketts SW 1975b Endometrial biopsy as a guide to diagnosis of endometrial pathology in the mare. *J Reprod Fertil Suppl* 341-345.

Ricketts SW & Alonso S 1991 The effect of age and parity on the development of equine chronic endometrial disease. *Equine Vet J* **23** 189-192.

Ricketts SW, Young A & Medici EB 1993 Uterine and clitoral cultures. In *Equine Reproduction*, pp. 266-302. Eds AO McKinnon & JL Voss. Philadelphia: Williams & Wilkins.

Riley SC, Gibson AH, Leask R, Mauchline DJ, Pedersen HG & Watson ED 2001 Secretion of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases into follicular fluid during follicle development in equine ovaries. *Reproduction* **121** 553-560.

Riley SC, Leask R, Chard T, Wathen NC, Calder AA & Howe DC 1999a Secretion of matrix metalloproteinase-2, matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases into the intrauterine compartments during early pregnancy. *Mol Hum Reprod* **5** 376-381.

Riley SC, Leask R, Denison FC, Wisely K, Calder AA & Howe DC 1999b Secretion of tissue inhibitors of matrix metalloproteinases by human fetal membranes, decidua and placenta at parturition. *J Endocrinol* **162** 351-359.

Riley SC, Thomassen R, Bae SE, Leask R, Pedersen HG & Watson ED 2004 Matrix metalloproteinase-2 and -9 secretion by the equine ovary during follicular growth and prior to ovulation. *Anim Reprod Sci* **81** 329-339.

Riley SC, Webb CJ, Leask R, McCaig FM & Howe DC 2000 Involvement of matrix metalloproteinases 2 and 9, tissue inhibitor of metalloproteinases and apoptosis in tissue remodelling in the sheep placenta. *J Reprod Fertil* **118** 19-27.

Roberts JM, Riemer RK, Bottari SP, Wu YY & Goldfien A 1989 Hormonal regulation myometrial adrenergic responses: the receptor and beyond. *J Dev Physiol* **11** 125-134.

Roberts RM, Bazer FW, Baldwin N & Pollard WE 1976 Progesterone induction of lysozyme and peptidase activities in the porcine uterus. *Arch Biochem Biophys* **177** 499-507.

Rodgers WH, Matrisian LM, Giudice LC, Dsupin B, Cannon P, Svitek C, Gorstein F & Osteen KG 1994 Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones. *J Clin Invest* **94** 946-953.

Roeb E, Schleinkofer K, Kernebeck T, Potsch S, Jansen B, Behrmann I, Matern S & Grotzinger J 2002 The matrix metalloproteinase 9 (mmp-9) hemopexin domain is a novel gelatin binding domain and acts as an antagonist. *J Biol Chem* **277** 50326-50332.

Rossdale PD & Ricketts SW 1980 Equine Stud Farm Medicine, 2nd ed. Philadelphia Lea & Febiger.

Rossdale PD & Silver M 1982 The concept of readiness for birth. *J Reprod Fertil Suppl* **32** 507-510.

Salamonsen LA 2003 Tissue injury and repair in the female human reproductive tract. *Reproduction* **125** 301-311.

Salamonsen LA, Butt AR, Hammond FR, Garcia S & Zhang J 1997 Production of endometrial matrix metalloproteinases, but not their tissue inhibitors, is modulated by progesterone withdrawal in an in vitro model for menstruation. *J Clin Endocrinol Metab* **82** 1409-1415.

Salamonsen LA & Woolley DE 1999 Menstruation: induction by matrix metalloproteinases and inflammatory cells. *J Reprod Immunol* **44** 1-27.

Salamonsen LA, Zhang J & Brasted M 2002 Leukocyte networks and human endometrial remodelling. *J Reprod Immunol* **57** 95-108.

Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H & Larjava H 1991 Transforming growth factor-beta 1 up-regulates type IV collagenase expression in cultured human keratinocytes. *J Biol Chem* **266** 11436-11441.

Samuel CA, Allen WR & Steven DH 1977 Studies on the equine placenta. III. Ultrastructure of the uterine glands and the overlying trophoblast. *J Reprod Fertil* **51** 433-437.

Sanderson N, Factor V, Nagy P, Kopp J, Kondaiah P, Wakefield L, Roberts AB, Sporn MB & Thorgeirsson SS 1995 Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions. *Proc Natl Acad Sci USA* **92** 2572-2576.

Schaefer L, Han X, Gretz N, Hafner C, Meier K, Matzkies F & Schaefer RM 1996 Tubular gelatinase A (MMP-2) and its tissue inhibitors in polycystic kidney disease in the Han:SPRD rat. *Kidney Int* **49** 75-81.

Schäffer MR, Tantry U, Ahrendt GM, Wasserkrug HL & Barbul A 1997 Stimulation of fibroblast proliferation and matrix contraction by wound fluid. *Int J Biochem Cell Biol* **29** 231-239.

Seki M, Uzuki M, Ohmoto H, Yoshino K, Maeda S, Kokubun S, Sakurai M & Sawai T 1995 Matrix metalloproteinase (MMP-9) in patients with rheumatoid arthritis. *Ryumachi* **35** 792-801.

Sevinga M, Vrijenhoek T, Hesselink JW, Barkema HW & Groen AF 2004 Effect of inbreeding on the incidence of retained placenta in Friesian horses. *J Anim Sci* **82** 982-986.

Sharp DC & Grubaugh 1987 Use of push-pull perfusion techniques in studies of gonadotrophin-releasing hormone secretion in mares. *J Reprod Fertil Suppl* **35** 289-96.

Sieme H, Schäfer T, Stout TA, Klug E & Waberski D 2003 The effects of different insemination regimes on fertility in mares. *Theriogenology* **60** 1153-64.

Silva LA, Gastal EL, Beg MA & Ginther OJ 2005 Changes in Vascular Perfusion of the Endometrium in Association with Changes in Location of the Embryonic Vesicle in Mares. *Biol Reprod* **72** 755-761.

Silver M, Barnes RJ, Comline RS, Fowden AL, Clover L & Mitchell MD 1979 Prostaglandins in maternal and fetal plasma and in allantoic fluid during the second half of gestation in the mare. *J Reprod Fertil Suppl* 531-539.

Simpson RB, Burns SJ & Snell JR 1975 Microflora in stallion semen and their control with a semen extender. *Proc Am Ass Equine Pract* **21** 255-261

Skinner JL, Riley SC, Gebbie AE, Glasier AF & Critchley HO 1999 Regulation of matrix metalloproteinase-9 in endometrium during the menstrual cycle and following administration of intrauterine levonorgestrel. *Hum Reprod* **14** 793-799.

Slauson DO & Cooper BJ 1990 Inflammation and repair. In *Mechanisms of disease. A textbook of comparative general pathology*, pp. 167-301. Eds DO Slauson & BJ Cooper. Williams & Wilkins.

Slusher SH, Freeman KP & Roszel JF 1984 Eosinophils in equine uterine cytology and histology specimens. *J Am Vet Med Assoc* **184** 665-670.

Squires EL 1993 Estrous detection. In *Equine reproduction*, pp. 186-195. Eds AO McKinnon & JL Voss. Philadelphia: Williams & Wilkins.

Ståhle-Bäckdahl M, Inoue M, Giudice GJ & Parks WC 1994 92-kD gelatinase is produced by eosinophils at the site of blister formation in bullous pemphigoid and cleaves the extracellular domain of recombinant 180-kD bullous pemphigoid autoantigen. *J Clin Invest* **93** 2022-2030.

Sternlicht MD & Werb Z 2001 How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* **17** 463-516.

Steven DH 1982 Placentation in the mare. *J Reprod Fertil Suppl* **31** 41-55.

Steven DH & Morriss G 1975 Development of the foetal membranes. In *Comparative placentation*, pp. 58-86. Ed DH Steven. London: Academic Press.

Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA & Goldberg GI 1995 Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem* **270** 5331-5338.

Strzemienski PJ, Do D & Kenney RM 1984 Antibacterial activity of mare uterine fluid. *Biol Reprod* **31** 303-311.

Sugamata M, Ihara T & Uchiide I 2005 Increase of activated mast cells in human endometriosis. *Am J Reprod Immunol* **53** 120-125.

Tamakoshi K, Kikkawa F, Nawa A, Maeda O, Kawai M, Sugamuma N, Yamagata S & Tomoda Y 1994 Different pattern of zymography between human gynecologic normal and malignant tissues. *Am J Obstet Gynecol* **171** 478-484.

Tan RJ, Fattman CL, Niehouse LM, Tobolewski JM, Hanford LE, Li Q, Monzon FA, Parks WC & Oury TD 2006 Matrix metalloproteinases promote inflammation and fibrosis in asbestos-induced lung injury in mice. *Am J Respir Cell Mol Biol* **35** 289-297.

Taylor PM, Silver M & Fowden AL 1992 Intravenous catheterisation of foetus and mare in late pregnancy: management and respiratory, circulatory and metabolic effects. *Equine Vet J* **24** 391-396.

Tekin S & Hansen PJ 2004 Regulation of numbers of macrophages in the endometrium of the sheep by systemic effects of pregnancy, local presence of the conceptus, and progesterone. *Am J Reprod Immunol* **51** 56-62.

Ter Heijden E, Chesnais JP & Hickman CG 1977 An efficient method of computing the numerator relationship matrix and its inverse matrix with inbreeding for large sets of animals. *Theor Appl Genet* **49** 237-241.

Tisdale JE, Follin SL, Ordelova A & Webb CR 1995 Risk factors for the development of specific noncardiovascular adverse effects associated with amiodarone. *J Clin Pharmacol* **35** 351-356.

Townson DH & Ginther OJ 1989 Size and shape changes in the preovulatory follicle in mares based on the digital analysis of ultrasonic images. *Anim Reprod Sci* **21** 63-71.

Traub-Dargatz JL, Salman MD & Voss JL 1991 Medical problems of adult horses, as ranked by equine practitioners. *J Am Vet Med Assoc* **198** 1745-1747.

Troedsson MH 1999 Uterine clearance and resistance to persistent endometritis in the mare. *Theriogenology* **52** 461-471.

Troedsson MH, deMoraes MJ & Liu IK 1993a Correlations between histologic endometrial lesions in mares and clinical response to intrauterine exposure with S*treptococcus zooepidemicus*. *Am J Vet Res* **54** 570-572.

Troedsson MH & Liu IK 1992 Measurement of total volume and protein concentration of intrauterine secretion after intrauterine inoculation of bacteria in mares that were either resistant or susceptible to chronic uterine infection. *Am J Vet Res* **53** 1641-1644.

Troedsson MH, Liu IK & Thurmond M 1993c Immunoglobulin (IgG and IgA) and complement (C3) concentrations in uterine secretion following an intrauterine challenge of Streptococcus zooepidemicus in mares susceptible to versus resistant to chronic uterine infection. *Biol Reprod* **49** 502-506.

Troedsson MH, Liu IK & Thurmond M 1993b Function of uterine and blood-derived polymorphonuclear neutrophils in mares susceptible and resistant to chronic uterine infection: phagocytosis and chemotaxis. *Biol Reprod* **49** 507-514.

Troedsson MH, Wistrom AO, Liu IK, Ing M, Pascoe J & Thurmond M 1993d Registration of myometrial activity using multiple site electromyography in cyclic mares. *J Reprod Fertil* **99** 299-306.

Troedsson MH, Loset K, Alghamdi AM, Dahms B & Crabo BG 2001 Interaction between equine semen and the endometrium: the inflammatory response to semen. *Anim Reprod Sci* **68** 273-8.

Tunón A-M, Nummijarvi A, Katila T, Magnusson U & Rodriguez-Martinez H 1999a Distribution of T cells in the endometrium of the mare 6 and 48 h after insemination. *Reprod Domest Anim* **34** 443-444.

Tunón AM, Rodriguez-Martinez H, Hulten C, Nummijarvi A & Magnusson U 1998 Concentrations of total protein, albumin and immunoglobulins in undiluted uterine fluid of gynecologically healthy mares. *Theriogenology* **50** 821-831.

Tunón AM, Rodriguez-Martinez H, Nummijarvi A & Magnusson U 1999b Influence of age and parity on the distribution of cells expressing major histocompatibility complex class II, CD4, or CD8 molecules in the endometrium of mares during estrus. *Am J Vet Res* **60** 1531-1535.

Tunon A-M, Katila T, Magnusson U, Nummijarvi A & Rodriguez-Martinez H 2000 T-cell distribution in two different segments of the equine endometrium 6 and 48 hours after insemination. *Theriogenology* **54** 835-841.

Vagnoni KE, Ginther OJ & Lunn DP 1995 Metalloproteinase activity has a role in equine chorionic girdle cell invasion. *Biol Reprod* **53** 800-805.

Vagnoni KE, Ginther OJ & Lunn DP 1996 Expression of major histocompatibility complex antigen and timing of invasion by equine chorionic girdle cells cultured on Matrigel. *Biol Reprod* **54** 219-223.

Vagnoni KE, Zheng J & Magness RR 1998 Matrix metalloproteinases-2 and -9, and tissue inhibitor of metalloproteinases-1 of the sheep placenta during the last third of gestation. *Placenta* **19** 447-455.

Vaillant B, Chiaramonte MG, Cheever AW, Soloway PD & Wynn TA 2001 Regulation of hepatic fibrosis and extracellular matrix genes by the th response: new insight into the role of tissue inhibitors of matrix metalloproteinases. *J Immunol* **167** 7017-7026.

Van Camp SD 1993 Uterine abnormalities. In *Equine reproduction*, pp. 392-396. Eds AO McKinnon & JL Voss. Philadelphia: Williams & Wilkins.

Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA & Opdenakker G 2002 Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol* **37** 375-536.

van Eldik P, van der Waaij EH, Ducro B, Kooper AW, Stout TAE & Colenbrander B 2006 Possible negative effects of inbreeding on semen quality in Shetland pony stallions. *Theriogenology* **65** 1159-1170.

Van Wart HE & Birkedal-Hansen H 1990 The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci USA* **87** 5578-5582.

Vandeplassche M, Podliachouk L & Beaud R 1970 Some aspects of twin-gestation in the mare. *Can J Comp Med* **34** 218-226.

Vidament M 2005 French field results (1985-2005) on factors affecting fertility of frozen stallion semen. *Anim Reprod Sci* **89**115-36.

Vincent AJ, Malakooti N, Zhang J, Rogers PA, Affandi B & Salamonsen LA 1999 Endometrial breakdown in women using Norplant is associated with migratory cells expressing matrix metalloproteinase-9 (gelatinase B). *Hum Reprod* **14** 807-15.

Walter I, Handler J, Miller I & Aurich C 2005 Matrix metalloproteinase 2 (MMP-2) and tissue transglutaminase (TG 2) are expressed in periglandular fibrosis in horse mares with endometrosis. *Histol Histopathol* **20** 1105-1113.

Walter I, Handler J, Reifinger M & Aurich C 2001 Association of endometrosis in horses with differentiation of periglandular myofibroblasts and changes of extracellular matrix proteins. *Reproduction* **121** 581-586.

Wan XS, Koch CJ, Lord EM, Manzone H, Billings PC, Donahue JJ, Odell CS, Miller JH, Schmidt NA & Kennedy AR 1995 Monoclonal antibodies differentially reactive with native and reductively modified Bowman-Birk protease inhibitor. *J Immunol Methods* **180** 117-130.

Watson ED 2000 Post-breeding endometritis in the mare. *Anim Reprod Sci* **60-61** 221-32.

Watson ED & Stokes CR 1988 Macrophage clearance of 125I-labelled polyvinyl pyrrolidone in the horse: effect of ovarian steroids and persistent endometritis. *Equine Vet J* **20** 421-423.

Watson ED & Stokes CR 1990 Effect of susceptibility to endometritis on specific antibody in the endometria of mares. *Theriogenology* **34** 39-45.

Watson ED, Stokes CR & Bourne FJ 1988 Concentrations of immunoreactive leukotriene B4 in uterine lavage fluid from mares with experimentally induced and naturally occurring endometritis. *J Vet Pharmacol Ther* **11** 130-134.

Watson ED, Stokes CR & Bourne FJ 1987a Cellular and humoral defence mechanisms in mares susceptible and resistant to persistent endometritis. *Vet Immunol Immunopathol* **16** 107-121.

Watson ED, Stokes CR & Bourne FJ 1987b Influence of administration of ovarian steroids on the function of neutrophils isolated from the blood and uterus of ovariectomised mares. *J Endocrinol* **112** 443-448.

Watson ED, Stokes CR, David JS, Bourne FJ & Ricketts SW 1987c Concentrations of uterine luminal prostaglandins in mares with acute and persistent endometritis. *Equine Vet J* **19** 31-37.

Weatherbys 2006 Conditions of entry to the General Stud Book. In *Supplement to return of mares 2006* Ed Weatherbys. Wellingborough: Weatherbys.

Westermarck J & Kahari VM 1999 Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J* **13** 781-792.

Whitwell KE & Jeffcott LB 1975 Morphological studies on the fetal membranes of the normal singleton foal at term. *Res Vet Sci* **19** 44-55.

Williamson P, Munyua S, Martin R & Penhale WJ 1987 Dynamics of the acute uterine response to infection, endotoxin infusion and physical manipulation of the reproductive tract in the mare. *J Reprod Fertil Suppl* **35** 317-325.

Williamson P, Munyua SJ & Penhale J 1989 Endometritis in the mare: A comparison between reproductive history and uterine biopsy as techniques for predicting susceptibility of mares to uterine infection. *Theriogenology* **32** 351-357.

Wingfield Digby NJ & Ricketts SW 1982 Results of concurrent bacteriological and cytological examinations of the endometrium of mares in routine stud farm practice 1978-1981. *J Reprod Fertil Suppl* **32** 181-185.

Wu J & Zern MA 2000 Hepatic stellate cells: a target for the treatment of liver fibrosis. *J Gastroenterol* **35** 665-672.

Xu P, Alfaidy N & Challis JR 2002 Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in human placenta and fetal membranes in relation to preterm and term labor. *J Clin Endocrinol Metab* **87** 1353-1361.

Yi CF, Gosiewska A, Burtis D & Geesin J 2001 Incorporation of Fluorescent Enzyme Substrates in Agarose Gel for in Situ Zymography. *Anal Biochem* **291** 27-33.

Yoshimura K, Dissanayake UB, Nanayakkara D, Kageyama I & Kobayashi K 2005 Morphological changes in oral mucosae and their connective tissue cores regarding oral submucous fibrosis. *Arch Histol Cytol* **68** 185-192.

Zent WW & Troedsson MH 1998 Postbreeding uterine fluid accumulation in a normal population of thoroughbred mares: A field study. *Proc Am Ass Equine Pract* **44** 65.

Zhang K, Gharaee-Kermani M, McGarry B, Remick D & Phan SH 1997 TNF-alpha-mediated lung cytokine networking and eosinophil recruitment in pulmonary fibrosis. *J Immunol* **158** 954-959.

Appendix I

Adobe Systems Incorporated
345 Park Avenue
San Jose
CA 95110-2704
USA

Amersham Biosciences UK Ltd
Pollards Wood
Nightingales Lanes
Chalfont Saint Giles
Bucks
HP8 4SP
United Kindom

Arnolds Veterinary Products / Dechra Veterinary Products
Cartmel Drive
Harlescott
Shrewsbury SY1 3TB
United Kingdom

Barbour Threads Ltd
Hilden Mill
Hilden
Lisburn BT27 4RR
Northern Ireland

Beckman Coulter Ltd
Oakley Court
Kingsmead Business Park
London Road
High Wickham
HP11 1JU
United Kingdom

Beecham Animal Health,
Division of GlaxoSmithKline PLC
980 Great West Road
Brentford
Middlesex TW8 9GS
United Kingdom

Bio-Rad Laboratories Ltd
Bio-Rad House
Maxted Road
Hemel Hempstead
Hertfordshire HP2 7DX
United Kingdom

Braun-Dexon
B. Braun Melsungen AG
Dörnbach 10
34283 Spangenberg
Germany

Calbiochem
Merck Biosciences Ltd
Boulevard Industrial Park
Padge Road
Beeston
Nottingham NG9 2JR
United Kingdom

Cecil Instruments Ltd
Milton Technical Centre
Cambridge
CB4 6AZ
United Kingdom

Critchley
138 Adderley Street
Auburn
NSW 2144
Australia

Dako
Dako Denmark A/S
Produktionsvej 42
DK-2600 Glostrup
Denmark

Graseby Medical Ltd
The Belfrey
Colonial Way
Watford WD2 4LG
Hertfordshire
United Kingdom

Icepharma hf.
Lynghálsi 13
110 Reykjavík
Iceland

Insight Biotechnology Ltd
PO Box 520
Wembley
Middlesex HA9 7YN
United Kingdom

Intervet UK Ltd
Walton Manor
Walton
Milton Keynes MK7 7AJ
United Kingdom

Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF
United Kingdom

Johnson & Johnson Ltd
1 Johnson & Johnson Plaza
New Brunswick
NJ 08933
USA

JVC (U.K.) Ltd
JVC House
JVC Business Park
Priestley Way
London NW2 7BA
United Kingdom

Kruuse
Jørgen KRUUSE A/S
Marslev Byvej 35
5290 Marslev
Denmark

Leica Microsystems GmbH
Ernst-Leitz-Strasse 17-37
35578 Wetzlar
Germany

Media Cybernetics, Inc
4340 East-West Hwy, Suite 400
Bethesda
MD 20814-4411
USA

Medical Wire & Equipment
Corsham
Wiltshire SN13 9RT
United Kingdom

Medion Diagnostics AG
Bonnstrasse 9
CH-3186
Düdingen
Switzerland

Menzel-Gläser
Gerhard Menzel
Glasbearbeitungswerk GmbH & Co. KG
Saarbrückener Str. 248
D-38116 Braunschweig
Germany

Merck Biosciences Ltd
Boulevard Industrial Park
Padge Road
Beeston
Nottingham NG9 2JR
United Kingdom

Molecular Probes, Inc.
PO Box 22010
Eugene
OR 97402-0469
USA

Pfizer Ltd
Ramsgate Road
Sandwich
Kent CT13 9NJ
United Kingdom

Sakura Finetek U.S.A., Inc.
1750 West 214th Street
Torrance
CA 90501
USA

Schering-Plough Animal Health
Breakspear Road South
Harefield
Uxbridge
Middlesex UB9 6LS
United Kingdom

Scion Corporation
82 Worman's Mill Court
Suite H
Frederick
Maryland 21701
USA

Sigma
Sigma-Aldrich Company Ltd.
The Old Brickyard
New Road
Gillingham
Dorset SP8 4XT
United Kingdom

Thermo Scientific
81 Wyman Street
Waltham
MA 02454
USA

Vector Laboratories
30 Ingold Road
Burlingame
CA 94010
USA

Appendix II

Gelatin zymography and reverse zymography reagents

Preparation of acrylamide gels

Reagents used in the preparation of gels are the same for zymography and reverse zymography

GELATIN ZYMOGRAPHY

Resolving gel

7.5% Acrylamide/Bis

Quantities sufficient for two gels 0.75mm thick

Water	3.85ml
Gelatin 10mg/ml water	1.0ml
Stock A buffer	2.5ml
10% SDS	100µl
10% Ammonium Persulfate	50µl
Acrylamide/Bis	2.5ml

Mix by gentle swirling, degas via desiccator vacuum for 5-10min

TEMED	5µl

Add TEMED, swirl gently to mix

Stacking gel

4% Acrylamide/Bis

Quantities sufficient for two gels

Water	3.05ml
Stock B buffer	1.25ml
10% SDS	50µl
10% Ammonium Persulfate	50µl
Acrylamide/Bis	0.65ml

Mix by gentle swirling, degas via desiccator vacuum for 5-10min

TEMED	5µl

Add TEMED, swirl gently to mix

REVERSE ZYMOGRAPHY

Resolving gel

12% Acrylamide/Bis

Quantities sufficient for two gels 0.75mm

Water	1.69ml
Gelatin 10mg/ml water	1.0ml
Stock A buffer	2.5ml
10% SDS	100µl
10% Ammonium Persulfate	50µl
Acrylamide/Bis	4.0ml
Solution A	0.66ml

Mix by gentle swirling, degas via desiccator vacuum for 5-10min

TEMED	5µl

Add TEMED, swirl gently to mix

Stacking gel

5% Acrylamide/Bis

Quantities sufficient for two gels

Water	2.89ml
Stock B buffer	1.25ml
10% SDS	50µl
10% Ammonium Persulfate	50µl
Acrylamide/Bis	0.812ml

Mix by gentle swirling, degas via desiccator vacuum for 5-10min

TEMED	5µl

Add TEMED, swirl gently to mix

Reagents

Resolving gel buffer – Stock solution A

Tris	18.3g	1.5M

Dissolve in ~50ml water, adjust pH to 8.8 with 5N HCl

Make up volume to 100ml with water.

Stacking gel buffer – Stock solution B

Tris	6.06g	0.5M

Dissolve in ~50ml water, adjust to pH 6.8 with 5N HCl

Make up volume to 100ml with water.

Also prepared 1:4 dilution with water, for use when storing gels overnight at 5°C.

Gelatin
10mg/ml w/v solution in water
Prepare ~25ml
Dissolve gelatin (Bovine Skin Type III, Bloom 225; Sigma)
Store in 1.2ml aliquots at 5°C ready for use.

Sodium Dodecyl Sulphate (SDS)
10% w/v solution in water
10g SDS dissolved in water, volume made up to 100ml
0.1ml of 10% in 10ml water for working solution

Ammonium Persulfate
10% w/v solution in water
50mg per 500µl of water
Make up freshly prior to preparing acrylamide gels.

Acrylamide
Acrylamide/Bis-Acrylamide 37.5:1
30% solution from Sigma, ready for use

sec-Butanol
Prepare a water saturated solution, equal volumes of butanol and water (50ml each), shake vigorously. Allow to settle and use top layer

Solution A
Supernatant from cultured BHK-21 cells expressing MMP-2 activity (supplied by Prof Dylan Edwards, University of East Anglia)

Application of samples and electrophoresis
The same reagents are used for gelatin zymography and reverse zymography

Sample application buffer

Glycerol	10ml
SDS	1g
Bromophenol Blue	20mg

Dissolved in 50ml water, stored in 1ml aliquots at 5°C

Running tank buffer

Tris	30.3gm	0.25M
Glycine	144g	1.9M
SDS	10g	1%

Dissolved in 1l water, should be ~ pH 8.3.

Dilute 1:10 with water for use, 500ml sufficient for Bio-Rad Mini Tank.

Washing and digesting gels

GELATIN ZYMOGRAPHY

Stock wash buffer x10 (TBS x10)

Tris	60.57g	0.5M
NaCl	90g	1.5M

Dissolve in ~750ml water, adjust to pH 8.0 with 5N HCl

Dilute 1:10 with water for use (TBS)

Triton-X-100 wash

2.5% Triton-X-100 in TBS 1:10

Digestion buffer

Tris	6.07g	50mM
NaCl	11.69g	0.2M
$CaCl_2$	735mg	5mM
$ZnCl_2$	1ml	1mM
Brij-35	660µl	30% solution

Dissolve in ~750ml water, adjust to pH 7.6 with 5N HCl
Make up volume to 1l with water, store at 5°C.

Zinc Chloride

ZnCl2	13.6mg	10mM

Dissolve in 10ml water
Dilute 1:10 with water to give 1mM
Store at 5°C

REVERSE ZYMOGRAPHY

Wash buffer/rinse buffer

Tris	6.05g	50mM
$CaCl_2$	735mg	5mM
Triton-X-100 2.5%	25ml	

Dissolve in ~750ml water, adjust to pH 7.5 with 5N HCl
Allow Triton-X-100 to dissolve completely and make up volume to 1l with water, store at 5°C.

Incubation buffer/digestion buffer

As rinse buffer but without the Triton-X-100.

Staining and destaining
Same staining and destaining solutions are used in gelatin zymography and reverse zymography.

Destaining solution
Methanol 300ml
Glacial acetic acid 100ml
Water 600ml

Staining solution
0.5% w/v Coomassie Brilliant Blue R250 in destaining solution
1g per 200ml of solution

Western blotting reagents

Reagents used in the recipes below are the same as used in gelatin zymography and reverse zymography.

Preparation of acrylamide gels

WESTERN BLOT
Resolving gel
7.5% Acrylamide/Bis
Quantities sufficient for two gels 1.5mm thick

Water	9.7ml
Stock A buffer	5ml
10% SDS	200µl
10% Ammonium Persulfate	100µl
Acrylamide/Bis	5ml

Mix by gentle swirling, degas via desiccator vacuum for 5-10min

TEMED	10µl

Add TEMED, swirl gently to mix

Stacking gel
4% Acrylamide/Bis
Quantities sufficient for two gels

Water	6.1ml
Stock B buffer	2.5ml
10% SDS	100µl
10% Ammonium Persulfate	100µl
Acrylamide/Bis	1.3ml

Mix by gentle swirling, degas via desiccator vacuum for 5-10min

TEMED	10µl

Add TEMED, swirl gently to mix

Application of samples and electrophoresis

Sample application buffer

Tris	375mg
SDS	1g
Dithiophreitol (DTT)	500mg
Bromophenol Blue	12.5mg

Dissolve Tris in ~20ml water, adjust pH to 6.75 with 5N HCl. Add in other chemicals, dissolve, make volume up to 25ml, store in 0.5ml aliquots at -20°C.

Running tank buffer
As for gelatin zymography/reverse zymography above

Washing and transferring gels

Stock wash buffer x10 (TBS x10)

Tris	60.57g	0.5M
NaCl	90g	1.5M

Dissolve in ~750ml water, adjust to pH 7.4 with 5N HCl.
Make up volume to 1l with water.

Dilute 1:10 with water for use and add:
Tween 20 500µl/1l 0.05% v/v (**TTBS**)

Membrane blocking buffer

BSA	5g (Bovine Serum Albumin; Sigma)

Dissolve in 100ml TTBS
Make up freshly ~30min prior to use, do not store.

Transfer/blotting buffer

Tris	3.03g	25mM
Glycine	14.4g	192mM
Methanol	200ml	

Dissolve salts in 800ml water, check pH (should be 8.1-8.4), add methanol. Do not alter, if below pH 8.0 remake. Store at 5°C.

In situ zymography reagents

Gelatin

10mg/ml w/v solution in water
Prepare ~25ml
Dissolve gelatin (Bovine Skin Type III, Bloom 225; Sigma)
Store in 1.2ml aliquots at 5°C ready for use.

DQ gelatin

10µg/ml solution in 10mg/ml bovine gelatin solution
DQ gelatin from pig skin, fluorescein conjugate (Molecular Probes) was dissolved in the bovine gelatin solution described above.